STUDY OF ENDOTHELIAL MORPHOGENESIS IN THREE-DIMENSIONAL COLLAGEN MATRICES

A Dissertation

by

SHIH-CHI SU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Genetics

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Approved by:

Chair of Committee,	Kayla J. Bayless
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ABSTRACT

Study of Endothelial Morphogenesis in Three-Dimensional Collagen Matrices.

(May 2011)

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Chair of Advisory Committee: Dr. Kayla J. Bayless

Sprouting angiogenesis is a multi-step process consisting of basement membrane degradation, endothelial cell (EC) activation, proliferation, invasion, lumen formation, and stabilization. Such complexity reveals that the orchestration of individual genes and multiple signaling pathways are required. To better understand the mechanisms that direct the transformation of adherent ECs on the surface of collagen matrices to multicellular invading sprouts, we analyzed differential gene expression with time using an *in vitro* model of EC invasion driven by the combination of sphingosine-1-phosphate (S1P) and angiogenic growth factors. Gene expression changes were confirmed by real-time PCR and Western blot analyses. In addition, we have undertaken a proteomic screen to dissect downstream targets of the S1P receptors that possibly regulate EC invasion. Gene silencing or overexpression were used to examine the involvement and role of downstream targets of S1P in EC invasion into three-dimensional collagen matrices.

We demonstrated that various cell adhesion molecule genes involved in adherens junction and cell-extracellular matrix (ECM) interactions were upregulated; whereas a set of genes associated with tight junctions were downregulated. Numerous genes encoding ECM proteins and proteases were induced, indicating that biosynthesis and remodeling of ECM is

indispensable for sprouting angiogenesis. Knockdown of a highly upregulated gene, A Disintegrin and Metalloproteinase with Thrombospondin-type repeats-1 (ADAMTS1), decreased invasion responses, confirming a role for ADAMTS1 in mediating EC invasion. Furthermore, differential expression of multiple members of the Wnt (wingless) and Notch pathways were observed. Functional experiments indicated that inhibition and activation of the Notch signaling pathway stimulated and inhibited EC invasion responses, respectively.

In addition, we identified annexin 2 as a regulator of endothelial morphogenesis. We observed that S1P triggered annexin 2 translocation from cytosol to plasma membrane and its association with vascular endothelial (VE)-cadherin. Moreover, annexin 2 depletion attenuated Akt activation, which was associated with increased phosphorylation of VE-cadherin and endothelial barrier leakage. Disrupting homotypic VE-cadherin interactions resulted in decreased Akt (but not Erk1/2) activation. Furthermore, expression of constitutively active Akt restored reduced EC invasion observed with annexin 2 and VE-cadherin knockdown. Collectively, we report that annexin 2 regulates endothelial morphogenesis through an adherens junction-mediated pathway upstream of Akt.

DEDICATION

This work is dedicated to my parents, sister, brother, and beloved wife for their continuous support that keeps me grounded and focused, and makes this possible.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Kayla Bayless for her guidance and support with regards to my graduate studies. Her patience and consideration have made everything go smoother, and her share of life experience during these years really encouraged my growth. I would also like to acknowledge Dr. Emily Wilson, Dr. Greg Johnson, and Dr. Steve Maxwell for serving as my committee members and for their helpful comments.

My appreciation is also extended to all current and former laboratory members. In particular, I would like to thank E. Adriana Mendoza for her invaluable assistance and Dr. Hyeong-il Kwak for creating a hilarious working environment and his technical support.

Finally, I am truly grateful for my family. This work would not be completed without them.

NOMENCLATURE

EC	Endothelial Cell
S1P	Sphingosine 1-phosphate
PCR	Polymerase Chain Reaction
ECM	Extracellular Matrix
ADAMTS1	A Disintegrin and Metalloproteinase with Thrombospondin-type repeats-1
TEER	Transendothelial Electrical Resistance
CAMs	Cell Adhesion Molecules
HUVEC	Human Umbilical Vein Endothelial Cell
VEGF	Vascular Endothelial Growth Factor
bFGF	basic Fibroblast Growth Factor
GPCR	G Protein- Coupled Receptors
MMP	Matrix Metalloproteinases
VE-cadherin	Vascular Endothelial-cadherin
AJ	Adherins Junction
TJ	Tight Junction

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1. INTRODUCTION

Angiogenesis, defined as the process where new blood vessels are formed from previously existing structures, is relatively rare in healthy adults, occurring only during the female reproductive cycle and wound healing (1). However, in certain pathological conditions, uncontrolled angiogenesis can occur, resulting in various diseases and cancer (1,2). The walls of blood vessels are composed of endothelial cells (ECs) and mural cells, which are embedded in an extracellular matrix (ECM). The process of angiogenesis involves EC activation, degradation of basement membrane, invasion, proliferation, lumen formation and stabilization. This process is regulated by a balance of pro- and anti-angiogenic molecules. Numerous studies both *in vivo* and *in vitro* have identified molecules and explain their related signaling pathways that regulate angiogenesis. This list includes, but is not limited to, growth factors, bioactive lipids, integrins, cell surface receptors, proteases and the ECM. These molecules and their related signals that modulate angiogenesis are complex and crosstalk with each other. Thus, discovery and comprehension of detailed molecular signaling pathways underlying angiogenic events including sprout initiation and lumen formation are key steps in understanding their roles during development, as well as during various pathological states.

Decades of research investigating the molecular basis of angiogenesis have identified a number of growth factor pathways that promote the formation of new blood vessels. Among them, vascular endothelial growth factor (VEGF) protein family is the most predominant regulator of both normal and pathologic angiogenesis. VEGF binds to its receptor, a family of receptor tyrosine kinases (VEGFR1, VEGFR2, VEGFR3), and regulates endothelial cell

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This dissertation follows the style of *the Journal of Biological Chemistry*.

migration, proliferation, and survival (3,4). Gene deletion of VEGF and the VEGFR1 and VEGR2 receptors in mice is lethal at embryonic stages, resulting in defects in cardiovascular abnormality and vasculogenesis (5-7). In addition, VEGF increases vascular permeability and has been implicated in malignant effusions (8,9). The permeability induced by VEGF leads to deposition of fibrin or other clotting proteins in the extravascular space, which subsequently facilitates angiogenesis (10,11). Recently, multiple reports have documented a role of VEGF in vessel branching where an endothelial tip cell leads the nascent vessel sprout at the forefront, while a trailing endothelial stalk cell elongates the stalk of this sprout (12-14). During branching morphogenesis, hypoxic cells upregulate VEGF which exists in a soluble form, and a VEGF gradient is established. The primary receptor for VEGF, vascular endothelial growth factor receptor 2 (VEGFR2) is expressed on both endothelial tip and stalk cells, but exerts distinct biological effects in these cells (13,15). In tip cells, VEGFR2 is abundant on filopodia, which extend into the direction of a VEGF gradient and induce migration. In stalk cells which are exposed to relatively lower levels of VEGF, the proliferative response is triggered (13). Genetic studies indicate that initiation of an angiogenic sprout depends on a gradient of VEGF, whereas elongation of a sprout is regulated by VEGF concentration (13,16). These findings, therefore, link VEGF to a novel object of anticancer therapies through targeting tumor vessels (17,18).

Fibroblast growth factor (FGF) protein family and their receptors are also highly relevant to angiogenesis (19), among which basic fibroblast growth factor (bFGF), also referred to as FGF2, is known to be important for new blood vessel growth during wound healing (20). Moreover, there is substantial crosstalk between receptors for FGF and VEGF, so FGF stimulates new vessel formation by similar mechanisms as does VEGF, including alteration of intercellular adhesion to modulate permeability as well as drive endothelial cell proliferation, survival, and migration (19,21). Although mice genetically deficient in FGF2 have no apparent

defects related to impaired angiogenesis (22,23), other evidence for a role of FGF2 in tumor angiogenesis has been established (19,24,25).

In addition to polypeptide growth factors, sphingosine-1-phosphate (S1P) is a biologically active sphingolipid that has been reported to induce angiogenesis under various conditions (26-30). The downstream signaling activated by S1P has been extensively studied. Cellular responses initiated by S1P are through one or more of its five known G protein- coupled receptors (GPCRs), $S1P_1$–$S1P_5$ (30). Knockout of the $S1P_1$ receptor in mice results in vascular defects, attributed to impairment of mural cell association with developing vasculature (31). In human umbilical vein endothelial cells (HUVECs), which express $S1P_1$ and $S1P_3$, it is known that S1P induced translocation of vascular endothelial (VE)-cadherin to endothelial junctions. VE-cadherin and β-catenin are the major determinants of adherens junctions in endothelial cells. This regulation of endothelial permeability by S1P required the activity of small GTPases Rho and Rac and was mediated by $S1P_1$ and $S1P_3$ (32). Moreover, cumulative evidence showed that S1P induced an increase in intracellular calcium concentration (33,34). This increase in calcium influx occurred due to the release of Ca^{2+} through activation of non-selective Ca^{2+} channels on plasma membrane and inositol 1,4,5-triphosphate (IP_3)-sensitive channels on endoplasmic reticulum (ER) (34-36). In addition to calcium homeostasis, S1P has also been shown to induce membrane ruffles and cell spreading of ECs (37,38). Interestingly, S1P signaling bears a complex crosstalk with that of VEGF as well. Two recent reports have shown that S1P can transactivate VEGF receptors in ECs, indicating that S1P, similar to various agonists of GPCRs, can activate growth factor receptors in the absence of added growth factors (37,39). Thus, numerous studies have implicated VEGF and S1P as significant regulators of endothelial cell migration, endothelial morphogenesis, angiogenesis, vascular permeability and maturation (32,40,41). Furthermore, the effects of S1P on different types of tumors prompted a flurry of

studies on the molecules involved in S1P synthesis (42). S1P is generated by conversion of sphingosine to S1P, which is catalyzed by sphingosine kinase (SPHK). SPHK exists as two isoforms, SPHK1 and SPHK2, both of which have been shown to be oncogenic and emerging as therapeutic targets for diverse tumors (42,43). Deciphering the complex interplay of S1P signaling inside and out and uncovering the clinical benefit of S1P, undoubtedly, appear to be an exciting area of lipid biology.

Another critical entity that regulates angiogenesis is the extracellular matrix (ECM). The ECM is well known for its ability to provide structural support for organs and tissues. At the cellular level, ECM supports cell layers in the form of basement membranes and provides a substrate for migration. There are hundreds of ECM proteins encoded in vertebrate genomes, and most ECM proteins are large, conserved, and composed of complex domains. The role of the ECM in cell adhesion and signaling to cells through integrin adhesion receptors has been well-studied (44-46). Moreover, many angiogenic growth factors, such as FGFs and VEGFs, bind avidly to components of ECM. Hence, a generally held view is that the ECM acts as a sink or reservoir of growth factors and may assist in establishing stable gradients of growth factors bound to the ECM. Such gradients of morphogens play vital roles in patterning developmental processes. It is also often proposed that growth factors can be released from the ECM by degradation of ECM proteins. The ECM degradation or remodeling is catalyzed by matrix metalloproteinases (MMP) that are prominently secreted by stromal cells (47) or by heparanase prominently expressed and secreted by tumor cells (48). This ECM degradation serves multiple purposes, which include the liberation of endothelial cells to migrate and proliferate from their cell-surface anchors (integrins), the liberation of sequestered growth factors (VEGF and bFGF) and the detachment of the pericytes that surround and support the blood vessels. Notably, these components released from ECM and basement membrane degradation serve as pro-angiogenic

factors. As the ECM undergoes MMP-mediated degradation and structural changes, cryptic domains of partially degraded collagens become exposed (49,50). These domains have been shown to provide important pro-angiogenic cues that were sequestered when the BM was fully assembled. A monoclonal antibody against denatured type IV collagen binds to one of these partially degraded and structurally altered ECMs *in vivo*, and suppresses angiogenesis (49,50). Another concept is that intrinsic domains within ECM proteins might act as ligands for canonical growth factor receptors. This suggestion raises from the observation that laminin contains multiple EGF-like domains, which might bind to EGF receptors and signal as solid-phase ligands (51). EGF-like domains from laminin (51,52), or tenascin (53) presented as soluble ligands can bind to EGF receptors and modulate downstream signaling. Thus, fragments of ECM proteins can be released by MMP-mediated proteolysis and act as soluble ligands, similar to the idea that matrix-bound growth factors can be released by ECM degradation to subsequently regulate angiogenic events.

The principle adhesion receptors used by endothelial cells to interact with ECM are integrins which play a critical role in regulating EC proliferation, migration, and survival (54,55). Integrins are heterodimeric receptors that mediate the attachment between a cell and its extracellular microenvironments but can also interact with cell surface and soluble ligands. Nascent integrin α and β subunits are paired in the endoplasmic reticulum to form functional heterodimers, which then traffic to the cell surface (56). Endothelial cells have been reported to express up to 10 different integrins depending on the location and activation state of the endothelial cell (55,57). The major integrins expressed on quiescent endothelial cells are α1β1, α2β1, α3β1, α5β1, α6β4, α6β1, αvβ3 and αvβ5. These integrins tend to be receptors for basal ECM components, such as collagen and laminin, with the exception of integrins αvβ5 and α5β1, which bind provisional matrix ligands such as vitronectin and fibronectin, respectively (55). The

α2β1 integrin is an endothelial receptor for collagen type I (58-60) and has been shown to be required for angiogenesis *in vivo* and *in vitro* (61,62), as have other integrins, including α1β1, αvβ3 and α5β1 (62-68). Among these, integrin αvβ3 has been shown to be particularly important for the angiogenic stimulation by VEGF and bFGF, indicating a crosstalk between integrins and growth factor receptors (69-74). The interactions of integrins with the ECM environment results in the induction of multiple cellular signaling pathways in vascular cells (75). These include activation of FAK (focal adhesion kinase), Ras, PI3K (phosphoinositide-3 kinase)-Akt signaling, MAPKs (mitogen-activated protein kinases), Src and Rac, Rho, and Cdc42 GTPases (76-79). Furthermore, recognition of ECM by some integrins such as αvβ3 results in the phosphorylation of integrin cytoplasmic tyrosine residues, which, in turn, promotes recruitment of intracellular adaptor proteins (80,81). This has provided important insights into the importance of integrins and growth factor receptors, which can associate to transduce downstream signals and promote vascular development in a number of angiogenesis-dependent processes. In addition, mice lacking the αv integrin, and therefore lacking αvβ3 and αvβ5 integrins, are lethal and have extensive blood vessel defects in the brain and intestinal tract (82). In contrast, although integrin αv gene knockout in mice is lethal, ablation of integrin β5 and/or β3 results in normal development of embryo and normal postnatal angiogenesis (83). Both integrin β5 and/or β3 null mice have extensive tumor angiogenesis *in vivo* (83). Since much of the other vasculature development appears to be normal, it clearly indicates that a compensatory mechanism does exist in these integrin knockout mice. Collectively, integrins are thus involved in the regulation of almost every facet of blood vessel maintenance, growth, and remodeling.

In addition to ECM ligation with integrin receptors, ECM remodeling is crucial for all aspects of vascular biology and tumor progression. The ECM can be remodeled by biosynthesis and proteolytic degradation. Cumulative evidence has shown that an intricate balance between

ECM proteinases and their inhibitors is critical for mediating diverse physiological events such as lineage decisions during embryogenesis, wound repair, cell migration, vascular stabilization and survival (84-87). Based on distinct domain structures, ECM proteolytic enzymes are divided into several protein families (88). The first group, the matrix metalloproteinases (MMPs) is a large family of highly conserved Zn-dependent endopeptidases (89). The second group consists of a cohort of serine proteases, such as thrombin, tissue plasminogen activator, urokinase and plasmin (90,91). The first two groups act generally as broad-spectrum proteases for major ECM degradation events and are principal participants in cancer metastasis (90,91). The third group, the bone morphogenetic protein 1(BMP-1)/tolloid-like metalloproteinases, has been linked to cellular differentiation and pattern formation through a proposed role in activating latent growth factors of the transforming growth factor β (TGF-β) superfamily (92,93). Finally, two large additional families, the ADAMs (a disintegrin and metalloproteinase domain) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are transmembrane glycoproteins with diverse roles in cell-cell adhesion and proteolysis (94-98).

Among them, MMPs have been recognized to play a central role in extracellular matrix turnover during angiogenesis (99,100). They comprise secreted MMPs and membrane-type MMPs (MT-MMPs). The activation of MMP is regulated by removal of their propeptide, complex formation with other proteins, and cellular internalization (101). Quiescent endothelial cells produce little or no active MMPs, but these proteases are strongly induced and subsequently activated in capillary sprouts during wound healing, inflammation and tumor growth (102-104) and in activated endothelial cells *in vitro* (105). In addition, MMP activities are controlled by a group of endogenous inhibitors known as Tissue Inhibitors of Metalloproteinases (TIMPs) and availability of specific substrates (104). Multiple studies have shown that MMPs, in particular MMP-1, MMP-2, MMP-9, MMP12, MMP-19, MMP-26, and

MT1-MMP play crucial roles in angiogenic events (99,100,102-104). MMP-2 and MMP-9, which are not only gelatinases but also cleave collagens present in the vascular basement membrane and the interstitium (106), appear to be the most well-studied MMPs in the field of angiogenesis. Although the MMP-2 knockout mouse exhibits no phenotype when unchallenged, MMP-2 is required for an activity-induced angiogenesis model, induced by electrical stimulation of rat skeletal muscle (107). Similarly, using transplantable tumor models in irradiated tissues, MMP-9 is found to be required for tumor vasculogenesis but not for angiogenesis (108). Moreover, MMP-9 has a distinct role in tumor angiogenesis, mainly through regulating the bioavailability of VEGF. MMP-9 has been shown to enable an angiogenic switch by making sequestered VEGF available for its receptor, VEGFR2, in pancreatic islet tumors (109), suggesting an angiogenic role for MMP-9 in coordination with a complex interplay of interconnected factors. In addition to secreted MMPs, the importance of MT1-MMP, a membrane-anchoring MMP, during angiogenesis is highlighted by the severe defects in skeletal development and angiogenesis leading to growth retardation and early death in MT1-MMP-deficient mice (110). MT1-MMP is generally considered as the most prominent player in pericellular proteolytic activity (101,111) and is essential in migration of cells in type-I collagen (112,113). There is ample evidence that MT1-MMP is present at the leading tip of invading cells (101,111,114,115), indicating its role in the initiation of angiogenesis. Like MMP-9, MT1-MMP not only cleaves extracellular matrix proteins but also mediates activation or modification of other pro-angiogenic factors, such as liberation of VEGF by proteolytic cleavage of connective tissue growth factor (CTGF) and shedding of soluble Semaphorin 4D from its membrane-bound form on tumor cells (116,117). The role of MMPs during angiogenesis appears more complex than originally envisaged; therefore, precise control of MMP activity is emerging as the novel strategy for targeting abnormal angiogenesis.

Another group of ECM proteinases highly involved in angiogenesis includes two related proteinase families, ADAM and ADAMTS. The ADAMTSs are closely related to the ADAM proteinases, on the basis of the similarity of their metalloproteinase domain to that of snake venom metalloproteinases (reprolysins) (118,119). Functionally, both ADAM and ADAMTS are involved in ectodomain shedding or activation of diverse cell surface molecules, including growth factors and adhesion receptors (120). However, unlike the mammalian ADAMs, which are mostly present as transmembrane proteins, the ADAMTSs are secreted molecules, some of which bind to ECM (119,121). ADAM-10 and ADAM-17 are involved in the release of ectodomains of membrane proteins, which results not only in removal of membrane receptors, but also in the generation of new biologically active molecules and in the activation of specific receptors involved in neovascularization, such as Notch, VEGFR2 and Tie-1 (122-127). In addition, ADAM-17 and ADAM-15 are found to associate with TIMP-3 to modulate endothelial sprouting events during angiogenesis (128). Furthermore, ADAM-15 colocalizes with vascular endothelial (VE)-cadherin, a major structural and regulatory protein of endothelial adherens junctions (129) and is required for angiogenesis during the development of retinopathy of prematurity *in vivo* (130). Nevertheless, its deficiency did not affect tumor angiogenesis in mice (130). ADAMTS-1 is dispensable for angiogenesis during murine development (131). However, in specific conditions ADAMTS-1 and -8 reduced VEGF-enhanced angiogenesis, such as in the chick chorioallantoic membrane and bFGF-enhanced angiogenesis in the cornea pocket (132).

Numerous evolutionarily conserved signaling pathways, including Notch, Wnt, and Hedgehog signaling pathways have been reported to modulate angiogenesis (133-138). Among them, Notch signaling is studied extensively on endothelial cell specification and tumor angiogenesis. There are four mammalian Notch genes, Notch1–Notch4, and five ligands, Jagged1 and Jagged2 (homologs of Drosophila Serrate-like proteins) and Delta-like 1 (DLL1),

DLL3 and DLL4. The Notch receptors and ligands, containing single-pass transmembrane domains, are expressed on the cell surface and, for that reason, Notch signaling is particularly important in mediating communication between adjacent cells. The initiation of Notch signaling occurs when the extracellular domain of the receptor engages ligand expressed on neighboring cells that are in close proximity. This leads to a cascade of enzymatic cleavages of Notch receptor and release of the extracellular domain, whereas the intracellular domain is released and then translocated to the nucleus where it interacts with CSL (CBF1, Su(H) and Lag-2) transcriptional repressors and converts them to transcriptional activators (139,140). In many cases, the cell that presents the ligand is a cell that does not have Notch signaling present, thus distinguishing two neighboring cells into one with ligand and low Notch signaling and the other with receptor and high Notch signaling. This idea is supported by the findings showing the role of Notch signaling in endothelial cell specification and the initiation of branching morphogenesis (12,137). Endothelial cells are heterogeneous in morphology, function, and gene expression. Depending on their state of activation, their position in the vascular bed, and the organ context, endothelial cells are specified toward particular roles (141). Notch signaling controls endothelial cell specification toward the arterial or venous phenotype in zebrafish and mouse (142-146). Targeted deletion of DLL1, DLL4, Notch1, Notch4, and Notch target genes, Hey1 and Hey2, in the mouse results in the deregulation of arterial and venous specification of endothelial cells, as well as in the deformation of arteries and veins (142-144,147-152). In addition, studies in the mouse retina and zebrafish indicate how Notch signaling, in coordination with VEGF signaling, regulates specification of endothelial tip versus stalk cells during new vessel formation and explain why endothelial cells do not move as a sheet, but instead form a perfused sprout (153-157). When cells are hypoxic, VEGF is upregulated and subsequently induces DLL4 expression. Because VEGF levels are highest at the vascular front, DLL4 is strongly expressed in tip cells,

while Notch signaling activity is greater in stalk cells (153,155). DLL4/Notch signaling from tip to stalk cells downregulates expression of VEGFR2 in stalk cells. High VEGFR2 signaling in the tip cell allows the cell to extend its filopodia and move ahead, while the VEGF migratory response in stalk cell is dampened. Hence, VEGF and Notch signaling engage in a reinforcing feedback loop that controls selection of tip cells. Formation of a vessel branch requires not only migration of tip cells but also proliferation of stalk cells. A downstream target of Notch, Nrarp (Notch-regulated ankyrin repeat protein) is found to counteract Notch signaling by destabilizing the Notch intracellular domain (NICD), and is expressed in stalk cells at branch points, leading to high proliferation (154). These findings regarding the role of DLL4/Notch signaling on vessel branching have been further tested by genetic and pharmacological loss-of-function and gain-of-function studies of DLL4 or Notch, in zebrafish and mice, both in the developing retina and in tumors. Indeed, neutralization of DLL4/Notch induces endothelial cells to adopt the migrating tip cell behavior and increases cell proliferation during stalk elongation, leading to supernumerary branches in a more dense vascular network (155-160). These reports document that endothelial cell-cell communication via the Notch signaling pathway contributes to functional vessel patterning and regulates tumor angiogenesis.

Another group of major players contributing to endothelial cell-cell communication in vessel organization is a myriad of transmembrane adhesion receptors that are responsible for maintaining the integrity of endothelial junctions. The endothelium is located at the inner side of all vessel types and is constituted by a monolayer of endothelial cells. Interendothelial junctions contain three complex junctional structures, including adherens junctions (AJs), tight junctions (TJs) and gap junctions (GJs). GJs are communication structures, which allow the passage of small molecular weight solutes between neighboring cells. However, TJs and AJs are formed by different molecules, but have common features. In both types of junctions, regardless of the cell

type, adhesion is mediated by transmembrane proteins that promote homophilic interactions and form a pericellular zipper-like structure along the cell border (161-166). TJs and AJs are mainly responsible for intercellular adhesion via the formation of actin filament-associated protein complexes along transmembrane adhesion sites (167) and highly relevant to the permeability and organization of blood vessels (167-169). Endothelial cells express cell type-specific transmembrane adhesion proteins, such as VE-cadherin at AJs (170) and claudin-5 at TJs (171). The restricted cell specificity of these components indicates that they might be needed for selective cell–cell recognition and/or specific functional properties of endothelial cells during angiogenesis.

VE-cadherin is the major determinant of endothelial adherens junctions. Regulation of its activity or presence at cell contacts is an essential step that controls the permeability of the blood vessel wall. VE-cadherin, similar to many other members of the cadherin family, is linked through its cytoplasmic tail to the AJ proteins p120, β-catenin and plakoglobin. β-catenin and plakoglobin bind to α-catenin, which interacts with several actin-binding proteins, including α-actinin, ajuba, zonula occludens-1 (ZO-1) and others, thereby forming a dynamic and huge protein complex at cell contacts (172). Endothelial cells possess several VE-cadherin-mediated mechanisms by which vascular permeability can be modulated. Such mechanisms focus on AJ organization and, in several cases, target VE-cadherin phosphorylation, cleavage and internalization. It has been reported that permeability-increasing agents such as histamine (173,174), tumor necrosis factor-α (TNFα) (175), platelet-activating factor (PAF) (176) and VEGF (177) induce the tyrosine phosphorylation of VE-cadherin and its binding partners, β-catenin, plakoglobin and p120. In several systems, the tyrosine phosphorylation of β-catenin reduces its affinity for the cadherin cytoplasmic tail and increases its turnover at junctions, thereby destabilizing AJs (178,179). Furthermore, the extent of tyrosine phosphorylation of VE-

cadherin is high in sparsely plated cells, but declined when cells reach confluence and establish stable intercellular junctions (180). Two *in vivo* studies that use organ extracts (181,182) have shown that VE-cadherin can be phosphorylated in angiogenic and ischemic conditions in mouse tissues. Therefore, it is proposed that the tyrosine phosphorylation of VE-cadherin and other components of AJs is associated with weak junctions and impaired barrier function. In addition to VE-cadherin phosphorylation, its turnover, including cleavage by MMPs (183,184) and a clathrin-dependent internalization (185), is implicated in vascular leakage as will. Notably, VE-cadherin also interacts with VEGF receptor 2 (186-188) and modulates signaling through phosphatidylinositol (PI) 3-kinase. This crosstalk between VE-cadherin and VEGF signaling plays a pivotal role in many aspects of angiogenesis. Moreover, disrupting the VE-cadherin gene in mouse embryonic stem (ES) cells is allowed to investigate its role in the development of vascular structures. Intriguingly, analyzing embryoid bodies developed from such ES cells reveals that the differentiation of endothelial cells is not impaired, yet they remain dispersed and fail to organize a vessel-like pattern (189). Mice deficient for VE-cadherin die at midgestation of vascular malformations (190). Defects are more severe in the extraembryonic vasculature. No capillary plexus is formed in the allantois, although inter-endothelial junctions do form as shown by electron microscopy. Thus, VE-cadherin seems dispensable for initial vasculogenesis but is required for subsequent remodeling and morphogenesis (190).

Like AJs, TJs are composed of both transmembrane and intracellular molecules and possess a strong impact on vascular permeability (167,168). Endothelial TJs specifically and highly express claudin-5, with a few exceptions (191). Unexpectedly, knockout of claudin-5 in mice does not result in a general breakdown of TJs but cause a selective increase in paracellular permeability of brain capillaries (171). This phenotype suggests a partial redundancy between claudin subtypes. Furthermore, claudin-5 expression is dependent on the expression levels and

interaction of VE-cadherin in endothelial cells (192). This finding places VE-cadherin upstream of claudin-5 in the establishment and maintenance of endothelial cell-cell junctions and further indicates not only a direct control of claudin-5 expression by VE-cadherin but also a functional relationship between AJs and TJs in vascular leakage or loss.

Other numerous and miscellaneous molecules are also shown as potential regulators of angiogenesis. Among them, annexin 2 is a multifunctional, Ca^{2+}-dependent membrane scaffold protein and has been implicated in developmental mechanisms, such as the establishment of epithelial polarity as well as the formation of new blood vessels (193,194). Annexin 2 was found to bind to the cytoskeletal proteins F-actin and non-erythroid spectrin two decades ago (195). Until now, it is believed that annexin 2 functions to organize the interface between the cytoplasm and plasma membrane by interacting with membrane phospholipids and actin filaments (196,197). Recent gene silencing studies indicate a role for annexin 2 in regulating endocytic and secretory events, as well as adherens junction and actin dynamics (198-201). In addition, annexin 2 has also been shown to be associated with and required for the formation of actin-rich tight junctions (202). Although annexin 2 has received much attention of its cellular or vascular functions, the mechanism by which annexin 2 translocates from cytosol to membranes and the signaling pathways downstream of annexin 2 during EC morphogenesis and other angiogenic events still remain to be elucidated.

As mentioned previously, sprouting angiogenesis is a multi-step process; thus, standard two-dimensional cell culture environments cannot completely reflect this dynamic process. Multiple approaches for studying endothelial sprouting have been developed to overcome this restriction, including Matrigel (203,204), collagen sandwich assays (205,206), endothelial cell outgrowth from polystyrene beads (207), and aortic ring outgrowth assays (208). Here, we utilize a three-dimensional (3-D) system of endothelial sprouting or invasion where S1P and angiogenic

growth factors, VEGF and bFGF synergistically induce robust endothelial morphogenesis in 3-D collagen matrices. The usefulness of S1P, VEGF, and bFGF in such model mimics a wound environment. Within wounded tissues, platelets deposit S1P and VEGF locally, while fibroblasts and other cells secrete bFGF (209-214). Thus, the coordination of S1P, growth factors, and the ligation to ECM are critical for endothelial cells to undergo angiogenesis during wound healing. By using this 3-D *in vitro* model, we mechanistically investigate the changes in gene expression and identify molecules that regulate the process. Data presented in this study indicate the coordinated regulation of molecules implicated in cell-cell and cell-matrix contacts, as well as in degradation and remodeling of ECM. Functional assays reveal that silencing of ADAMTS1, a highly upregulated gene, decreases invasion responses, confirming a role for ADAMTS1 in mediating EC invasion. Inhibition and activation of the Notch signaling pathway, whose members are differentially regulated during EC invasion, stimulate and dampen EC sprouting responses, respectively. Moreover, we show that specific knockdown of annexin 2 in ECs decreased invasion responses and attenuated Akt activation, which is associated with impaired integrity of endothelial adherens junctions, indicating a functional requirement for annexin 2 during EC morphogenesis.

2. MATERIALS AND METHODS*

2.1 Endothelial Cell Culture

Human umbilical vein endothelial cells (ECs), passage 3-6 (Lonza, Cambrex, MA) were passaged once weekly and cultured on gelatin-coated (1 mg/mL) tissue culture flasks in medium 199 (M199) containing 100 µg/mL heparin (Sigma), 0.4 mg/mL lyophilized bovine hypothalamic extract (Pel-Freeze Biologicals) (215), 15% fetal bovine serum (Lonza), antibiotics and antimycotics (216). Collagen Type I was isolated from tendons of one rat tail by incubation with gentle agitation in 150 mL 0.1% acetic acid for 1 week. Supernatants were lyophilized, weighed, and resuspended in 0.1% acetic acid at 7.1 mg/mL and stored at 4°C. Collagen matrices were prepared at 2.5 mg/mL with 1 µM S1P (Avanti Polar Lipids, Alabaster, AL) as reported previously (217). Gels (25 µL) were added to half area (A/2) 96-well plates (Costar) and allowed to equilibrate for 45 minutes at 37°C with 5% CO_2. Cells were fed 24 hours before the beginning of each experiment. For invasion assays, confluent flasks of ECs were washed with 1X HEPES-buffered saline, trypsinized, and counted. The final cell pellet was resuspended at a density of 30,000 cells per 50 µL in M199 and allowed to attach for 30 minutes. Growth media contained RSII, 50 µg/mL ascorbic acid (Sigma), and 40 ng/mL VEGF and bFGF (R&D Systems). The RSII was added from a sterile 250X stock containing 500 µg/mL bovine serum albumin (BSA) (Sigma), 5 µg/mL human holo-transferrin (Sigma), 5 µg/mL insulin (Sigma),

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4.28 µg/mL Sodium oleate (Sigma), and 5 ng/mL Sodium selenite (Sigma).

2.2 Gene Profiling Studies and Data Analysis

Three replicate experiments were performed, where invading cells were collected at 0, 6, 12 and 18 hours. Collagen matrices were digested with 50 µg collagenase (Sigma) each at 37°C for 5 minutes before being transferred into 8 ml of 1X M199 and centrifuged at 350 g for 5 minutes. RNA was extracted using an RNA extraction kit according to manufacturer's instructions (Qiagen). RNA was eluted with 50 µL of nuclease-free water (Ambion). Total RNA was submitted to the Texas A&M University Center for Environmental and Rural Health for quality analysis using an Agilent Technologies 2100 Bioanalyzer. RNA was generated into biotin-labeled cRNA via a modified Eberwine RNA amplification protocol. The labeled cRNA was applied to the bioarray (GE Amersham CodeLink Human Whole Genome) and incubated for 18 hours then washed, stained, and scanned. The array images were processed using CodeLink's system software. Three replicates for each time point (0, 6, 12 and 18 hours) were performed. Data were averaged and compared to time zero data. Each data entry was analyzed by GeneSpring software at the Texas A&M Laboratory for Functional Genomics and considered for further analysis only if a 1.7 fold or greater elevation was observed.

2.3 Invasion Quantification

For quantifying the average numbers of invading cells per standardized field, conditioned media were removed and invasion samples were fixed with 3% glutaraldehyde (Sigma) in PBS overnight at 4°C. Cultures were stained with 0.1% Toluidine blue (Sigma) containing 30% methanol (Fisher). Alternatively, cultures were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 10 minutes, stained with 1.09 µM DAPI (Molecular Probes), and

stored in the dark prior to quantification. Eyepieces mounted with a vertical displaying a 10x10 grid which covers an area of 6.25 mm^2, 1 mm^2, and 0.25 mm^2 at 4X, 10X and 20X, respectively, were used for quantifying average numbers of invading cells per standardized field. For quantifying invasion distance and lumen diameter, photographs of invading cells were taken from a side view. Invasion distance and lumen diameter were measured digitally using QCapture software (Olympus/Leeds) and pixel values were converted to microns. For each data set, three or four separate fields from each treatment were recorded and averaged.

2.4 Real-Time PCR

An AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene) was used to prepare cDNA isolated from invading ECs. Primers were designed using Beacon Designer software such that amplicons were 180-220 bp. Primer sequences are listed in Appendix A. Primers were validated by real-time PCR using a 5-fold serial dilution (125-0.04 ng/reaction) of untreated HUVEC cDNA and water as a non-template control followed by agarose gel electrophoresis, to determine the amplification efficiency, specificity, and to rule out primer dimers. Reactions were analyzed on a BioRad iCycler iQ Multicolor Real-Time PCR Detection system using iQ SYBR Green Supermix (Bio-Rad Lab, Hercules, CA). Each real-time PCR reaction contained 0.5 ng/μL of cDNA and 400 nM of each primer in a 25 μL reaction volume. The reaction was initiated at 94°C for 1.5 minutes, followed by 40 two-step amplification cycles consisting of 15 seconds denaturation at 95°C and 45 seconds annealing/elongation at 60°C. A final dissociation stage was run to generate a melting curve for verification of amplicon specificity. Assays were performed in triplicate against 3 independent preparations of cDNA. For each reaction a threshold cycle (Ct) was observed in the exponential phase of amplification and the quantification of relative expression levels was achieved using standard curves for both the

target and a constitutively expressed gene, GADPH, whose expression changed less than 1.15 fold.

2.5 Immunoblotting and Immunofluorescence

For immunoblotting, total lysates of invading cultures were prepared by removing conditioned media and transferring collagen gels containing invading ECs into boiling 1.5X Laemmli sample buffer containing 2% β-mercaptoethanol at 95°C for 10 minutes. Samples were separated using SDS-PAGE gels and transferred to PVDF (Millipore). Antibodies against the following proteins were used for detection: claudin-5 (35-2500, Invitrogen), integrin α2 (611016, BD Biosciences), integrin αv (611012, BD Biosciences), ADAMTS1 (ab39194, Abcam, Cambridge, MA), Dkk2 (ab38594, Abcam), DAAM1 (M05, clone 5D3, Novus Biologicals, Littleton, CO), Delta-like 4 (NB600-892, Novus Biologicals), actin (CP01, Calbiochem), annexin II (AF3928, BD Transduction Laboratories), β2-microglobulin (M8523, Sigma), GAPDH (ab8245, Abcam), pan-cadherin (ab16505, Abcam), connexin43 (C6219, Sigma), calpain S1 (ab28237, Abcam), Akt (9272, Cell Signaling), phosphor-Akt (Ser473, 4060, Cell Signaling), Erk2 (sc-153, Abcam), phosphor-p44/42 MAP kinase (p-Erk, 9101, Cell Signaling), Rac1 (ARC03, Cytoskeleton), Cdc42 (610928, BD Transduction Laboratories), VE-cadherin (sc-52751, Santa Cruz Biotechnology), PECAM1 (sc-1505, Santa Cruz Biotechnology), phosphor-VE-cadherin (Y731, 441145G, Invitrogen), β-catenin (sc-7199, Santa Cruz Biotechnology), anti-FLAG M2 (F3165, Sigma), N-cadherin (sc-7939, Santa Cruz Biotechnology), GFP (218), and HRP-conjugated secondary antibodies (Dako). Immunoblots were conducted on Immobilon membranes (Millipore). Densitometric analysis of blots was performed using ImageJ software. For immunofluorescence analyses, cell cultures (whole mount) were fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.5% Triton X-100. Nonspecific binding

was blocked by incubation with PBS containing 1% goat serum, 1% BSA, 0.2% sodium azide, and 0.1% Triton X-100. Following incubation with an anti-FLAG M2 monoclonal antibody (Sigma; 1:100 dilution) for 2 hours and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch; 1:50 dilution) for 1 hour, samples were mounted on glass slides with an anti-fading, aqueous mounting medium (Biomeda, Foster city, CA). For ECs cultured on glass coverslips, samples were rinsed with PBS and fixed in 100% cold methanol at -20°C for 15 minutes. Nonspecific binding was blocked by incubation with PBS containing 1% BSA, 1% goat serum and 0.1% Triton X-100 overnight at 4°C. Following incubation of primary antibodies for detection: mouse anti-VE cadherin (sc-52751, Santa Cruz Biotechnology), mouse anti-claudin-5 (35-2500, Invitrogen), or rabbit anti-VE-cadherin (ab71285, Abcam), 1:100 dilution for 1 hour. After washing three times, Alexa Fluor secondary antibodies (Invitrogen; 1:200 dilution) were added for 1 hour in PBS containing 1% BSA, 10% goat serum and 0.1% Triton X-100. Samples were washed three times and mounted on glass slides with an anti-fading, aqueous mounting medium (Biomeda, Foster City, CA).

2.6 siRNA Transfection

siGENOME SMARTpool human ADAMTS1, ADAMTS4, ADAM17, and GAPDH control siRNAs were obtained from Dharmacon (Lafayette, CO). Cells were seeded into 25 cm^2 flasks at 40-50% confluence. The following day, cells were washed three times with serum- and antibiotic-free DMEM and transfected with 200 nM siRNA in 2.7 mL antibiotic-free DMEM using 20 uL siPORT Amine (Ambion, Austin, TX). Flasks were aspirated and supplemented with antibiotic-free growth media at 8 hours post-transfection. This transfection procedure was repeated 2 days after the first transfection. After the second transfection, cells were fed and

allowed to recover for 30 hours prior to testing in invasion assays and verification of gene knockdown.

2.7 Gamma-Secretase Inhibition

Cells were resuspended at a density of 40,000 cells per 50 μL in M199 containing RSII and indicated concentrations of γ-secretase inhibitor IX (565770, Calbiochem) or vehicle control (DMSO), incubated at 37°C with 5% CO_2 for 20 minutes prior to seeding on collagen gels with 100 nM S1P. For γ-secretase inhibition, growth media contained RSII, ascorbic acid, VEGF, FGF-2, and indicated concentrations of γ-secretase inhibitor. Cultures were fixed at 24 hours to quantify invasion.

2.8 GTP-Rac1/GTP-Cdc42 Pull-Down Assay

Rho GTPase activation assay during EC invasion in 3-D collagen matrices was performed as reported previously (219). In brief, EC cultures were extracted at indicated conditions using cold detergent lysis buffer of 1% Triton X-100 in Tris-buffered saline, pH 8.0, containing Complete Protease Inhibitor Cocktail (Roche Diagnostics), 150 μg/μL high-purity collagenase (Sigma), and 100 nM GTPγS (Calbiochem). Lysates were incubated at 4°C for 60 minutes to dissolve collagen and clarified by centrifugation at 15,000 X g for 15 minutes at 4°C. Supernatants were incubated with GST-PAK-PBD protein agarose beads (Cytoskeleton) for 45 minutes at 4°C. The beads were washed four times. Bound active Rho GTPases were detected using Western blot analyses.

2.9 Plasmid Constructs, Gene Expression, and Gene Silencing

Recombinant lentiviral vector encoding an enhanced green fluorescent protein (EGFP) was previously described (220) and was a kind gift from Dr. George E. Davis (Columbia, MO). Notch1 intracellular domain (N1ICD) (amino acids 1770-2556), Notch4 intracellular domain (N4ICD) (amino acid 1476-2003), and Dll4 were amplified by PCR from HUVEC cDNA and subcloned into pFLAG-CMV-5a (Sigma). The constructs were sequenced and tested for expression of the C-terminal FLAG-tagged N1ICD, N4ICD, and Dll4 in HEK293 cells using anti-FLAG M2 antibodies (Sigma). FLAG-N1ICD, FLAG-N4ICD, and FLAG-Dll4 were amplified by PCR using pFLAG-CMV-5a clones as the templates and subcloned into pENTR4 (Invitrogen). Subsequently, genes with FLAG sequences were subcloned into pLenti6/V5-DEST using the Gateway system (Invitrogen). For constructing the lentiviral vector that encodes annexin 2 fused to a C-terminal green fluorescent protein (ANXA2-GFP) and a C-terminal FLAG tag (ANXA2-FLAG), annexin 2 was amplified by PCR from EC cDNA and subcloned into pEGFP-N2 (Clontech) and pFLAG-CMV-5a (Sigma), respectively. The constructs were sequenced and tested for expression of the C-terminal GFP-tagged annexin 2 or C-terminal FLAG-tagged annexin 2 in HEK293 cells. ANXA2-GFP and ANXA2-FLAG were amplified by PCR using pEGFP-N2-based ANXA2-GFP vector and pFLAG-CMV-5a clone, respectively, as the templates and subcloned into pENTR4 (Invitrogen). Subsequently, ANXA2-GFP and ANXA2-FLAG were subcloned into pLenti6/V5-DEST using Gateway system (Invitrogen). For constructing the lentiviral vector that encodes myrAkt, insert was amplified by PCR using pcDNA3 Myr HA Akt1 (Addgene) as the template and subcloned into pENTR4 (Invitrogen). Subsequently, myrAkt was subcloned into pLenti6/V5-DEST using Gateway system (Invitrogen) and sequenced. ECs were transduced with indicated constructs using ViraPower Lentiviral Expression Systems (Invitrogen) according to the manufacturer's instructions. For gene silencing,

ECs were infected with pLKO.1-puro lentiviral vectors encoding shRNAs against human annexin A2, β2-microglobulin, GFP, VE-cadherin, and PECAM1 (Sigma, sequences are available in Appendix B) using ViraPower Lentiviral Expression System (Invitrogen). For annexin A2 and VE-cadherin knockdown, findings were reproduced by two distinct shRNAs. Invasion assays were conducted 3-7 days following infection. Alternatively, cells infected with lentiviruses were propagated under 1μg/mL blasticidin selection (for gene expression) or 0.25μg/mL puromycin selection (for gene silencing).

2.10 Microscopy and Imaging

For visualization of invasion responses, photographs of invading cells were taken from top view and side view using an Olympus CKX41 microscope equipped with a Q color 3 Olympus camera and 20X objective. For immunofluorescence imaging, top-viewed photographs (whole mount) were taken under a Nikon Eclipse TE2000U fluorescence inverted microscope equipped with a CCD camera and Metamorph software (Universal Imaging Corp.), and side-viewed photographs were captured by Stallion digital imaging workstation at the Image Analysis Laboratory, Texas A&M University.

2.11 Subcellular Fractionation

ECs were serum-starved for 4 hours and treated with 1 μM S1P or the combination of 40 ng/ml VEGF and bFGF for 0, 30 and 60 minutes. EC membranes were prepared by incubating the cells in a lysis buffer [20 mM HEPES, pH 7.4, 20 mM NaCl, 1.5 mM $MgCl_2$, 250 mM sucrose, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, Complete Protease Inhibitor Cocktail (Roche Diagnostics) and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific)]. Lysates were passed through a 25G needle 10 times using a 1 mL syringe and kept on ice for 20

minutes. After homogenization, lysates were centrifuged at 1000 X g for 5 minutes at 4°C to remove unbroken cells. The supernatants were collected and centrifuged at 150,000 X g for 30 minutes at 4°C. The resulting supernatants corresponded to cytoplasmic fractions. Pellets, corresponded to the membrane fractions, were resuspended in lysis buffer containing 0.5% NP-40 and analyzed by Western blotting or utilized for immunoprecipitation.

2.12 Immunoprecipitation

Protein samples were incubated with 2 μg of indicated antisera (rabbit anti-VE-cadherin, ab71285, Abcam, rabbit anti-FLAG, F7425, Sigma, and normal rabbit IgG, 2729, Cell Signaling) in 500 μL of lysis buffer containing 0.5% NP-40 overnight at 4°C with gentle shaking. 20 μL of Protein G Dynabeads (Invitrogen) were added to each sample, incubated for another 2 hours, and washed extensively. Magnetic beads were suspended in 1X Laemmli sample buffer containing 2% β-mercaptoethanol for Western blot analyses.

2.13 FITC-Dextran Permeability Assay

Endothelial permeability was assessed by quantifying diffusion of FITC-dextran across endothelial monolayers. 50,000 ECs were seeded on top of gelatin-coated Transwell chambers for 24-well plates (0.4-μm pore size; Falcon) and transduced with lentiviruses for 8 hours. Subsequently, medium containing lentiviruses was replaced with phenol red-free culture media, and cells were allowed to grow for 72 hours, or until a monolayer was formed. Cells were serum-starved for 6 hours in phenol red-free M199 (Invitrogen) prior to adding 25 μL of 20 μg/μL FITC-dextran (70 kDa; Sigma) into the upper chamber. The amount of FITC-dextran across endothelial monolayers was assessed 1 hour later by taking 100 μL aliquots from the outer

chambers, and then measured using an Infinite M200 Microplate Reader (Tecan) with excitation/emission at 485/530 nm.

2.14 Transendothelial Electrical Resistance (TEER)

For measuring the TEER, 50,000 ECs were seeded on top of gelatin-coated Transwell inserts for 24-well plates (0.4-μm pore size; Costar) and transduced with lentiviruses for 8 hours. Subsequently, medium containing lentiviruses was replaced with culture medium. TEER was monitored using an Evometer (World Precision Instruments) fitted with a Chopstick electrode every 24 hours after lentiviral administration. Results were normalized by the area of the monolayer, and the background TEER of blank inserts was subtracted from the TEER of the EC monolayer.

3. RESULTS*

3.1 Characterization of an in vitro *Endothelial Cell (EC) Invasion Assay*

The studies performed here utilize a 3D model of endothelial invasion which mimics angiogenesis (217). In these experiments, ECs are seeded as a monolayer on collagen matrices (Fig. 1A). No appreciable invasion occurred under control conditions, with sphingosine-1-phosphate (S1P) alone or with growth factors (GF) alone. However, combining S1P, with bFGF and VEGF, the invasion events occur rapidly, within 16 hours (Figure 1B). Quantification of these data (Figure 1C) illustrates that the combination of S1P, and VEGF and bFGF, potently stimulates invasion responses. On the basis of these data, all subsequent experiments were carried out in the presence of S1P, VEGF and bFGF. Further quantification with time reveals that ECs invade into the collagen matrices forming sprouting structures containing lumens. Photographs taken of fixed cultures viewed from the top (Fig. 1D) and side (Fig. 1E) illustrate that invasion initiated at 6 hours, with sprouts increasing at 12 hours and extending further by 18 hours. The invading structures recapitulate angiogenesis, forming lumens (Fig. 1F) lined by multiple cells (Fig. 1G). Quantification of the distance invaded over time reveals that ECs migrate through the collagen matrix at a linear rate from 0 to 48 hours (Fig. 1H). In addition, quantification of lumen formation in these assays shows that lumen formation does not initiate with sprouting morphogenesis, but is delayed, initiating between 12 and 16 hours (Fig. 1I).

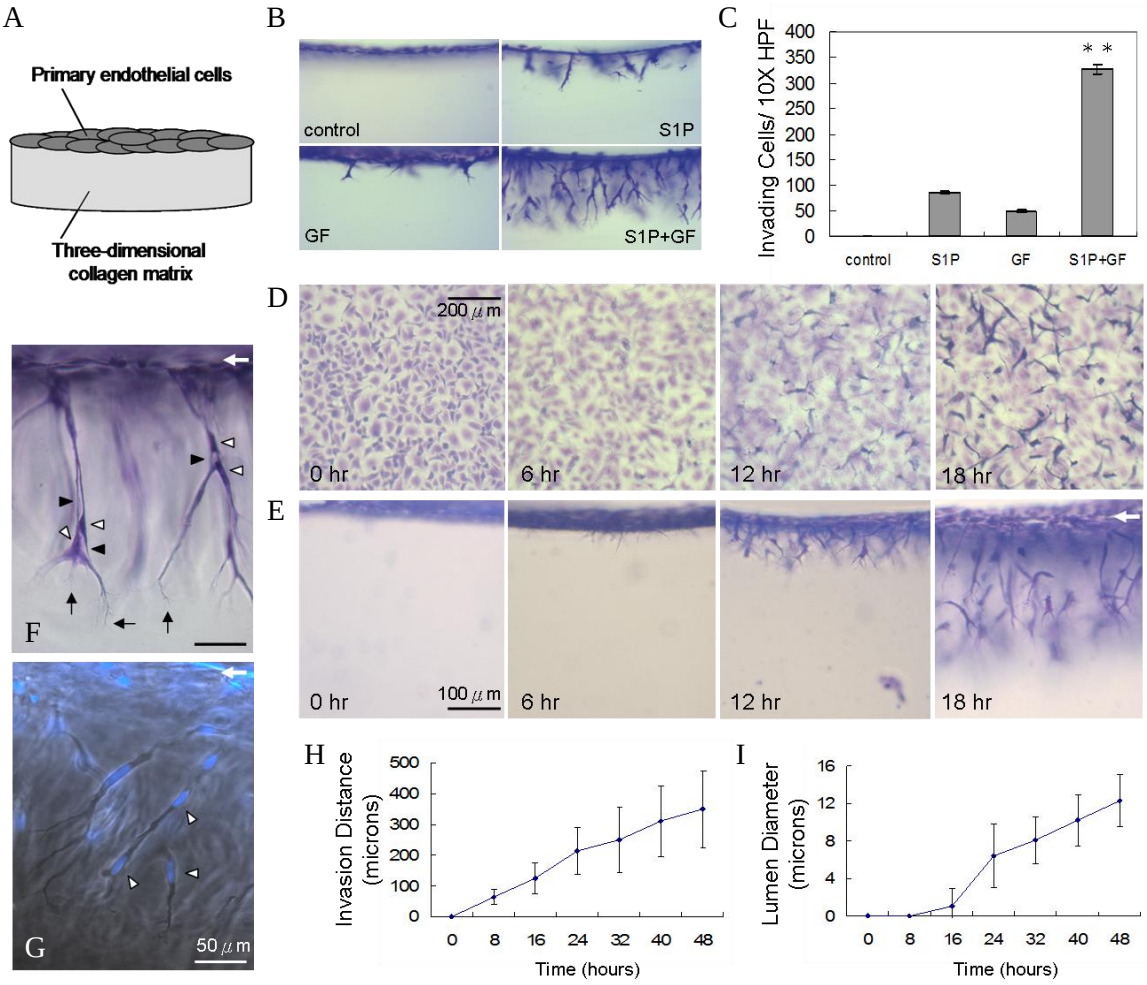


FIGURE 1 **Assay System to Study Primary EC Invasion in 3-D Collagen Matrices**. (A) Illustration of invasion system. (B) Photographs illustrating that S1P and angiogenic growth factors (GF), VEGF and bFGF, synergize to stimulate invasion. Cultures were allowed to proceed for 24 hours under control conditions (No S1P or GF), 1μM S1P alone, GF alone (40 ng/ml VEGF and bFGF) or S1P and GF. (C) Quantification of invasion responses observed in (B). Data presented are average numbers of invading cells per standardized field ± SEM. (n=3 fields). (D-E) Photographs depicting EC invasion over time. Cells were seeded on the surface of 3-D collagen matrices and allowed to attach for 30 minutes prior to addition of growth factors as described in MATERIALS AND METHODS section. Cultures were fixed at 0, 6, 12, 18 hour and stained with toluidine blue prior to imaging from (D) surface and (E) side view. (F-G) Photographs illustrating that invading structures are multi-cellular. Cultures were fixed at 24 hour, stained with toluidine blue (F) or DAPI (G). Phase contrast and DAPI images were overlayed (E). White arrowheads indicate nuclei; black arrowheads, areas of lumen formation (light open areas); black arrows, extended peripheral processes at the leading edge of invading structures. (H-I) Quantification of EC invasion rates. Cells were allowed to invade for the times indicated, fixed, stained, and quantified for invasion distance (H) and lumen diameter (I). Data are presented as mean ± SEM; n = 100 (cells). In all panels, white arrows indicate original monolayer where primary ECs were seeded.

3.2 Microarray Analysis of EC Gene Expression during S1P and Angiogenic Growth Factor-Induced Invasion in 3-D Collagen Matrices

To gain a more complete understanding of the mechanisms that direct the transformation of adherent ECs on the surface of a collagen matrix to multicellular invading structures, gene expression profiling studies were performed at various times. Total RNA from invading cells was isolated at 0, 6, 12 and 18 hours and subjected to microarray analyses as described in the MATERIALS AND METHODS section. The zero-hour time point contained cells that were seeded onto collagen matrices lacking S1P and growth factors, thus our microarray analyses aim to compare gene expression changes with the advancement of invading sprouts. Of the 30,000 human genes screened, 440 were significantly upregulated and 742 were downregulated at least 1.7 fold. A partial listing of these genes is shown in Tables 1 and 2, respectively. Although multiple and diverse genes were differentially regulated, the focus of this report is on the expression of genes related to ECM interactions, proteolysis, cell-cell communication and regulation of morphogenesis, or cell shape changes.

3.3 Differential Expression of Cell Adhesion Molecules (CAMs) and Extracellular Matrix (ECM) Genes during EC Invasion

Our data reveal that a series of genes that control endothelial cell-cell junctions and ECM recognition are systematically regulated during invasion. The invasion cultures consist of two populations of ECs, those that invade into the collagen matrix and those that stay behind. The decision to leave the monolayer and invade is likely associated with alterations in signaling between cell-cell contacts. Fitting with this idea, a set of genes that regulate tight junction (TJ) integrity was observed (Table 2). These downregulated genes include connexin 40 (CX40), connexin 45 (CX45), zona occludens 2 (ZO2), claudin-5 (CLDN5), claudin-11 (CLDN11), and

TABLE 1 **Genes Upregulated during S1P- and Growth Factor-Induced Invasion**.

0	6	12	18hr	
				Angiogenesis-Related Genes
1	17	1.4	0.8	chemokine (C-C motif) ligand 1 (CCL1)
1	6.1	4.4	1.8	SH2 domain protein 2A (SH2D2A)
1	3.2	2.5	1.1	vascular endothelial growth factor receptor 1 (Flt1)
1	3.1	2.7	1.2	c-fos induced growth factor (VEGF D)
1	3.0	2.5	1.5	sprouty homolog 1 (SPRY1)
1	8.5	4.5	1.4	insulin-like growth factor 1 (IGF1)
1	5.0	3.0	2.0	fibroblast growth factor 16 (FGF16)
1	2.2	1.7	1.5	FGF receptor substrate 3 (FRS3)
1	1.7	1.3	0.9	platelet derived growth factor D (PDGFD)
1	1.7	2.0	2.0	chemokine (C-X-C motif) receptor 4 (CXCR4)
1	1.6	1.7	2.2	proprotein convertase subtilisin/kexin type 1 (PCSK1)
1	1.5	1.8	1.4	endothelial cell-specific molecule 1 (ESM1)
1	1.4	1.4	1.7	PDGF receptor, α (PDGFRA)
				Extracellular Matrix
1	10.8	12.0	6.3	secreted phosphoprotein 1 (osteopontin, SPP1)
1	10.1	6.5	4.9	statherin (STATH)
1	5.5	5.9	4.0	decorin (DCN), transcript variant B
1	4.1	3.2	3.0	laminin, gamma 2 (LAMC2)
1	2.8	2.9	2.0	collagen, type I, alpha 2 (COL1A2)
1	2.2	2.0	1.8	nidogen 2 (NID2)
				Cell-Cell Contacts/ Surface Receptors
1	3.2	2.5	1.0	protocadherin 10 (PCDH10)
1	6.6	6.9	3.6	protocadherin 17 (PCDH17)
1	7.3	3.4	1.6	MADCAM1
1	3.5	3.5	4.4	CUB domain-containing protein 1 (CDCP1)

TABLE 1 **continued.**

0	6	12	18hr	
1	4.4	3.1	2.5	anthrax toxin receptor 2 (ANTXR2, CMG2)
1	3.1	2.5	1.9	ANTXR1, transcript variant 3
1	2.9	2.4	1.1	podocalyxin-like (PODXL)
1	2.1	1.4	0.7	av integrin (CD51)
1	1.8	1.7	1.1	a2 integrin (CD49)
1	2.7	0.8	0.4	E selectin (SELE)
				Cell Shape Changes (Small GTPases)
1	14.3	8.7	2.2	Rho GTPase activating protein 6 (ARHGAP6)
1	8.2	7.9	4.7	pleckstrin 2 (PLEK2)
1	4.1	3.1	2.1	ninein, transcript variant 3 (NIN)
1	3.8	1.9	0.6	docking protein 5 (DOK5), transcript variant 1
1	3.3	2.8	1.9	RAP1A
1	2.9	2.1	1.4	GRB2-associated binding protein 1 (GAB1)
1	2.4	1.4	1.0	nebulette (NEBL)
1	2.2	2.0	1.7	PH-like domain, family B, member 1 (PHLDB1)
1	2.2	2.0	0.8	desmuslin (DMN), transcript variant A
1	1.9	1.5	1.2	Rho-related BTB domain containing 1 (RHOBTB1)
1	1.9	2.0	1.8	Dok-like protein (FLJ22570)
1	2.4	1.5	1.0	tubulin, beta polypeptide paralog (TUBB2B)
1	2.2	2.0	0.7	desmuslin (DMN)
1	2.4	1.9	1.3	RAS guanyl releasing protein 3 (RASGRP3)
				G-protein Coupled Receptors
1	12.3	1.8	1.6	regulator of G-protein signalling 2 (RGS2)
1	5.8	5.2	2.2	adenosine receptor A3 (ADORA3)
1	4.6	4.8	3.0	G protein-coupled receptor 98 (GPR98)
1	2.6	2.4	2.0	protease activated receptor 2 (PAR2)

TABLE 1 **continued.**

0	6	12	18hr	
1	2.8	2.8	1.0	leupaxin (LPXN)
1	2.8	2.7	1.6	guanine nucleotide binding protein (G protein) gamma transducing activity polypeptide 2 (GNGT2)
1	2.5	2.6	1.7	RGS20
1	2.4	2.2	1.3	RGS3, transcript variant 3
				Signaling
1	3.0	3.5	2.9	ligand of numb-protein X (LNX)
1	2.1	2.2	1.8	receptor-interacting Ser-Thr kinase 3 (RIPK3)
1	7.8	7.4	3.2	protein tyrosine phosphatase, non-receptor type 7
1	7.2	3.2	0.7	Down syndrome critical region gene 1 (DSCR1)
1	7.0	6.8	1.8	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian) (MYCN)
1	5.8	5.3	2.1	AD026 protein (AD026)
1	12	3.9	0.5	dickkopf homolog 2 (DKK2)
1	5.0	4.5	1.7	dishevelled associated activator of morphogenesis 1 (DAAM1)
1	2.6	2.5	1.5	mitogen-activated protein kinase 13 (MAPK13)
1	2.1	1.8	0.8	caspase recruitment domain family, member 11 (CARD11)
1	2.0	1.5	1.0	phosphatidic acid phosphatase type 2A (PPAR2A)
1	2.0	2.0	2.1	phosphatidylinositol transfer protein, membrane-associated 1 (PITPNM1)
1	9.7	4.9	1.6	TNF superfamily, member 11 (TNFSF11, RANKL)
1	3.7	2.7	3.2	TNF receptor-associated factor 1 (TRAF1)
1	2.0	2.8	1.5	TNF superfamily, member 18 (TNFSF18)
				Proteolysis
1	4.8	5.4	7.2	protease, serine, 2 (trypsin 2) (PRSS2)
1	4.1	1.8	0.7	ADAMTS1
1	2.5	0.5	0.3	ADAMTS5
1	3.5	3.5	1.5	tissue plasminogen activator (tPA)
1	2.7	1.9	1.9	urokinase plasminogen activator (uPA)

TABLE 1 **continued.**

0	6	12	18hr	
1	2.5	2.3	2.4	latexin (LXN)
1	2.2	1.8	1.2	matrix metalloproteinase-10 (MMP-10)
1	2.2	2.3	2.1	neuroserpin (SERPINI 1)
1	2.0	1.9	1.5	carboxypeptidase M (CPM)
1	1.9	1.6	1.3	alpha-2-macroglobulin (A2M)
1	1.7	2.2	1.7	cathepsin K
				Transcription Factors
1	6.1	2.1	1.3	zinc finger protein 167 (ZNF167), transcript variant 1
1	3.7	1.1	0.5	v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS)
1	2.2	1.6	0.9	microphthalmia-associated transcription factor (MITF), transcript variant 3
1	3.2	2.2	2.0	transcription factor 7 (TCF7), transcript variant 2
1	2.1	1.7	0.8	v-ets erythroblastosis virus E26 oncogene hom. 1 (ETS1)
1	4.3	2.7	1.4	interferon regulatory factor 6 (IRF6)
1	2.3	2.2	1.5	PR domain containing 1, with ZNF domain (PRDM1)
1	2.5	2.3	2.1	PR domain containing 2, with ZNF domain (PRDM2)
1	2.2	2.0	1.8	ret finger protein 2 (RFP2), transcript variant 2
				Ion Channels
1	14.3	6.7	1	K^+ voltage-gated channel shaker-related subfamily, beta member 1 (KCNAB1)
1	12.4	8.6	4.5	K^+ intermediate/small conductance Ca^{2+}-activated channel, subfamily N, member 2 (KCNN2)
1	3.7	3.6	1.7	K^+ intermediate/small conductance Ca^{2+}-activated channel, subfamily N, member 3 (KCNN3)
1	2.4	1.8	1.3	K^+ large conductance Ca^{2+}-activated channel, subfamily M, beta member 4 (KCNMB4)
				Inflammation & Immunity
1	10.6	6.8	4.8	neutrophil cytosolic factor 4 (NCF4)
1	9.8	5.3	2.4	chemokine (C-C motif) receptor-like 1 (CCRL1)
1	4.3	2.7	1.4	interferon regulatory factor 6 (IRF6)
1	4.1	4.1	2.8	Thy-1 cell surface antigen (THY1)

TABLE 1 **continued.**

0	6	12	18hr	
1	2.9	1.8	1.2	Interleukin receptor 3, beta (IL3RB, CSFR2B)
1	2.8	2.7	1.3	cyclooxygenase-1 (COX-1, PTGS1)
1	3.3	3.0	1.5	interferon tau-1 (IFNE1)
1	2.5	2.9	3.8	interleukin 16 (IL16)
1	2.3	1.0	0.3	phospholipase A2, group IVA (PLA2G4A)
1	2.3	2.2	1.7	interferon stimulated gene 20kDa (ISG20)
1	2.0	1.6	1.3	immunodeficiency virus type I enhancer binding protein 2 (HIVEP2)
				Miscellaneous
1	11	3.7	1.0	stanniocalcin 1 (STC1)
1	5.5	3.9	2.0	kynureninase (L-kynurenine hydrolase) (KYNU)
1	5.4	8.1	2.4	asparagine synthetase (ASNS), transcript variant 1
1	4.2	4.0	3.2	myozenin 2 (MYOZ2)
1	3.1	3.0	1.3	aldo-keto reductase family 1, member B10 (AKR1B10)
1	3.0	2.7	2.0	proopiomelanocortin (POMC)
1	2.7	2.4	1.7	pregnancy specific beta-1-glycoprotein 11 (PSG11),
1	2.6	2.0	1.3	DnaJ (Hsp40) homolog, subfamily C, member 12
1	2.5	1.9	1.0	NADPH oxidase 4 (NOX4)
1	2.3	2.5	0.5	growth arrest and DNA-damage-inducible, gamma (GADD45G)
1	2.1	1.9	1.2	GRINL1A complex upstream protein (Gup1)
1	2.1	2.3	2.0	apolipoprotein L, 4 (APOL4), transcript variant a
1	2.5	2.9	1.2	neurobeachin (NBEA)
1	2.2	1.6	1.0	exostoses (multiple) 1 (EXT1)

TABLE 2 **Genes Downregulated during S1P- and Growth Factor-Induced Invasion**.

0	6	12	18hr	
				Angiogenesis-Related Genes
1	0.39	0.32	0.27	netrin 4 (NTN4)
1	0.72	0.40	0.20	heparin-binding epidermal growth factor-like (HB-EGF)
1	0.32	0.29	0.25	basic fibroblast growth factor (bFGF)
1	0.37	0.26	0.19	endothelial PAS domain protein 1 (EPAS1)
1	0.50	0.43	0.40	nucleolin (NCL)
				Cell-Cell Contacts/ Surface Receptors
1	0.16	0.07	0.06	connexin 40 (GJA5)
1	0.41	0.38	0.28	connexin 45 (GJA7)
1	0.53	0.53	0.45	zona occludens 2 (ZO-2)
1	0.20	0.15	0.15	claudin 23 (CLDN23)
1	0.34	0.37	0.42	claudin 5 (CLDN5)
1	0.42	0.35	0.19	claudin 11 (CLDN11)
1	0.36	0.37	0.33	semaphorin 6B (SEMA6B)
				Cell Shape Changes (Small GTPases)
1	0.35	0.31	0.77	paralemmin 2 (PALM2, AKAP2)
1	0.47	0.48	0.28	v-ral simian leukemia viral oncogene homolog B (RALB)
1	0.17	0.12	0.05	Rho family GTPase 1 (Rho6)
1	0.54	0.45	0.33	RAB30 (Ras oncogene family)
1	0.58	0.53	0.38	ADP-ribosylation factor 6 (ARF6)
1	0.58	0.51	0.34	Ras-related GTP binding B (RRAGB)
1	0.59	0.53	0.56	CDC42 effector protein 3 (CDC42EP3)
1	0.70	0.63	0.40	RAB21 (member Ras oncogene family)
1	0.74	0.66	0.53	Rap1 GTP-GDP dissociation stimulator 1 (RAP1GDS1)

TABLE 2 **continued.**

0	6	12	18hr	
1	0.49	0.64	0.22	ras-related C3 botulinum toxin substrate 2 (RAC2)
1	0.15	0.15	0.20	mysosin VIIA and Rab-interacting protein (MYRIP)
1	0.34	0.32	0.28	dystrophin (DMD)
1	0.48	0.39	0.56	Ras association and pleckstrin homology domains 1(RAPH1)
1	0.56	0.44	0.45	diaphanous homolog 3 (DIAPH3)
1	0.30	0.26	0.28	peanut-like 2 (PNUTL2), transcript variant 1
1	0.54	0.49	0.14	kinesin family member 22 (KIF22)
1	0.43	0.30	0.16	catenin, alpha-like 1(CTNNAL1)
				Apoptosis
1	0.32	0.27	0.22	Bcl2-like 11 (BCL2L11)
1	0.35	0.25	0.19	myeloid cell leukemia sequence 1 (MCL1)
				G-protein Coupled Receptors
1	0.23	0.16	0.11	G protein-coupled receptor 126 (GPR126)
1	0.39	0.37	0.33	regulator of G-protein signaling 4 (RGS4)
1	0.47	0.48	0.51	GPCR116
1	0.50	0.41	0.32	guanine nucleotide binding protein, alpha 14 (GNA14)
				Signaling
1	0.43	0.26	0.22	bone morphogenic protein 2 (BMP2)
1	0.31	0.35	0.38	phospholipase C, gamma 2 (PLCG2)
1	0.36	0.45	0.39	ephrin-A1 (EphA1)
1	0.46	0.50	0.31	ephrin-B1 (EphB1)
1	0.47	0.39	0.24	sprouty homolog 2 (SPRY2)
1	0.41	0.41	0.33	MAP kinase-interacting serine/threonine kinase 2 (MKNK2)
1	0.49	0.41	0.38	adenylate kinase 3 (AK3)
1	0.51	0.38	0.25	protein phosphatase 1D magnesium-dependent, delta (PPM1D)
1	0.14	0.13	0.12	SMAD7

TABLE 2 **continued.**

0	6	12	18hr	
1	0.30	0.17	0.10	SMAD6
1	0.30	0.26	0.23	NF-κB inhibitor, alpha (NFKBIA)
1	0.44	0.48	0.31	protein phosphatase 5, catalytic subunit (PPP5C)
1	0.05	0.05	0.04	SNF1-like kinase (SNF1LK)
1	0.55	0.37	0.34	interleukin-1 receptor-associated kinase 3 (IRAK3)
1	0.29	0.15	0.17	BIRC3 (baculoviral IAP repeat-containing 3) - binds TNF receptor assoc. factor (TRAF) 1&2
1	0.36	0.39	0.47	delta-like 4 (DLL4)
1	0.40	0.18	0.23	jagged 1 (JAG1)
1	0.47	0.42	0.22	transducer of regulated cAMP response element binding protein 2 (TORC2)
1	0.29	0.29	0.25	AXIN1 upregulated 1 (AXUD1)
1	0.34	0.35	0.42	Hedgehog interacting protein (HHIP)
1	0.53	0.54	0.43	Frizzled homolog 4 (FZD4)
				Proteolysis
1	0.29	0.29	0.35	HERC5
1	0.40	0.37	0.53	carboxypeptidase A3 (CPA3)
1	0.77	0.77	0.34	cathepsin S
				Transcription Factors
1	0.50	0.43	0.40	cAMP responsive element binding protein-like 2 (CREB3L2)
1	0.45	0.37	0.26	hairy and enhancer of split 1 (HES1)
1	0.07	0.06	0.05	HES related with YRPW motif 1 (HEY1)
1	0.09	0.08	0.07	forkhead box F1 (FOXF1)
1	0.32	0.27	0.29	forkhead box A1 (FOXA1)
1	0.22	0.14	0.05	GATA binding protein 6 (GATA6)
1	0.35	0.31	0.30	GATA binding protein 2 (GATA2)
1	0.31	0.30	0.42	GATA binding protein 3 (GATA3)
1	0.18	0.15	0.14	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATC1)

TABLE 2 **continued.**

0	6	12	18hr	
1	0.18	0.11	0.09	nuclear receptor subfamily 4, group A, member 3 (NR4A3)
1	0.41	0.21	0.08	Kruppel-like factor 2 (KLF2)
1	0.47	0.40	0.27	Kruppel-like factor 2 (KLF3)
1	0.31	0.09	0.07	Kruppel-like factor 4 (KLF4)
1	0.30	0.29	0.22	jun B protooncogene (JUNB)
1	0.29	0.26	0.20	basic helix-loop-helix domain containing, class B,2 (BHLHB2)
1	0.29	0.26	0.19	GDNF-inducible zinc finger protein 1 (GZF1)
1	0.37	0.29	0.21	nuclear receptor subfamily 2, group F, member 1 (NR2F1)
1	0.21	0.23	0.30	zinc finger protein 145 (ZNF145)
1	0.37	0.43	0.25	Max binding protein (MNT)
1	0.44	0.39	0.32	nuclear factor I/B (NFIB)
1	0.27	0.31	0.20	interferon regulatory factor 1 (IRF1)
1	0.51	0.35	0.20	nuclear factor, interleukin 3 regulated (NFIL3)
1	0.27	0.50	0.17	period homolog 1 (PER1)
1	0.47	0.50	0.39	TIMELESS
1	0.48	0.41	0.24	HOXA5 (homeo box A5)
1	0.55	0.51	0.36	HOXB5 (homeo box B5)
1	0.49	0.40	0.44	TGF-beta inducible early growth response (TIEG)
1	0.49	0.40	0.38	snail homolog 1 (SNAI1)
				Cell Cycle Regulation & Cell Division
1	0.36	0.34	0.32	cell division cycle 6 homolog (CDC6)
1	0.40	0.31	0.10	kinetochore protein Spc25 (Spc25)
1	0.40	0.55	0.18	growth arrest-specific 2 like 1 (GAS2L1)
1	0.39	0.32	0.19	non-SMC condensin I complex, subunit H (NCAPH)
1	0.42	0.31	0.11	chromosome condensation protein G (NCAPG)
1	0.45	0.31	0.08	cyclin A2 (CCNA2)
1	0.70	0.53	0.48	cyclin-dependent kinase inhibitor 1A (p21)

TABLE 2 **continued.**

0	6	12	18hr	
1	0.43	0.28	0.18	cyclin-dependent kinase inhibitor 1B (p27)
1	0.35	0.30	0.35	RA-regulated nuclear matrix-associated protein (DTL)
				Ion Channels
1	0.18	0.16	0.14	solute carrier family 30, zinc transporter member 1 (SLC30A1)
1	0.35	0.27	0.24	potassium channel tetramerization domain containing 12 (KCTD12)
				Inflammation & Immunity
1	0.19	0.15	0.05	pentaxin-related gene, rapidly induced by IL-1 beta (PTX3)
1	0.28	0.14	0.05	interleukin1 receptor-like 1 (IL1RL1)
1	0.57	0.20	0.32	interleukin-8 (IL8)
1	0.63	0.47	0.40	chemokine orphan receptor 1 (CMKOR1)
				Metabolism/Lipid Biosynthesis
1	0.13	0.11	0.07	TCDD-inducible poly (ADP-ribose) polymerase (TIPARP)
1	0.30	0.26	0.15	3-hydroxy-3-methylglutaryl-CoenzymeA synthase 1 (soluble HMGCS1)
1	0.33	0.30	0.32	isopentenyl-diphosphate delta isomerase (IDI1)
1	0.35	0.33	0.25	stearoyl-CoA desaturase (delta-9-desaturase, SCD)
1	0.36	0.36	0.28	dehydrogenase/reductase (SDR family) member3
1	0.37	0.36	0.27	steroidogenic acute regulatory protein (STARD4)
1	0.32	0.17	0.08	aldehyde dehydrogenase 1, family member A1 (ALDH1A1)
				DNA Replication/Repair
1	0.18	0.18	0.18	minichromosome maintenance deficient 10 (MCM10)
1	0.31	0.32	0.26	minichromosome maintenance deficient 4 (MCM4), transcript variant 2
1	0.09	0.08	0.05	growth arrest and DNA-damage inducible, beta (GADD45B)
1	0.39	0.31	0.11	topoisomerase II alpha (TOP2A)
1	0.35	0.26	0.18	ASF1 anti-silencing function 1 homolog B (ASF1B)
1	0.37	0.34	0.33	polymerase (DNA directed), epsilon 2 (POLE2)

TABLE 2 **continued.**

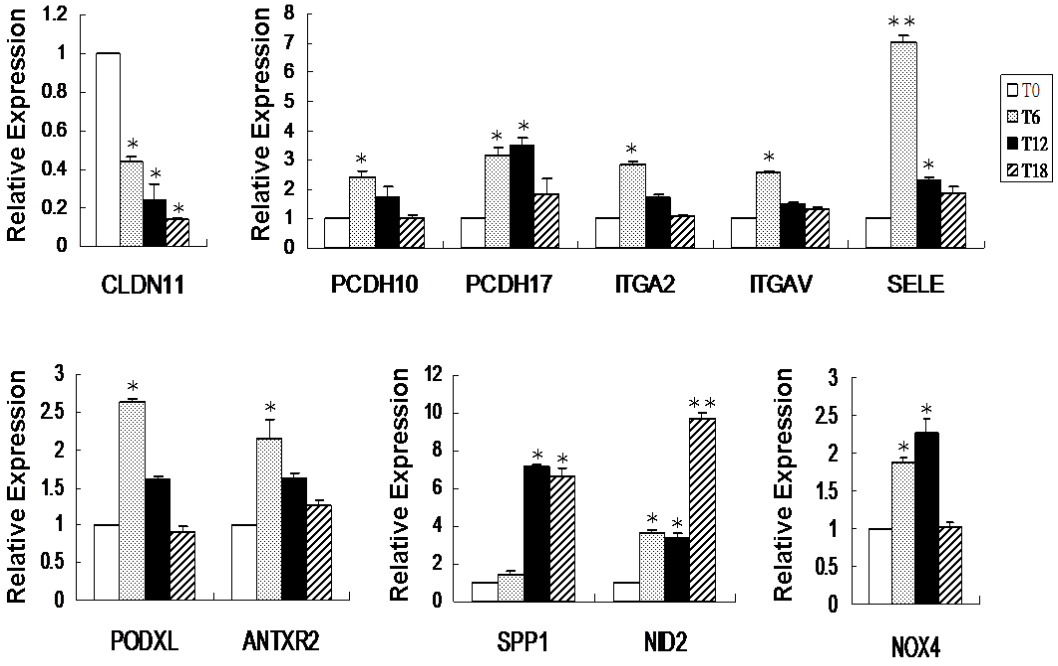
0	**6**	**12**	**18hr**	
				Miscellaneous
1	0.06	0.04	0.08	inhibin, beta B (INHBB)
1	0.05	0.04	0.03	cytochrome P450 family 26 subfamily B, polypeptide 1 (CYP26B1)
1	0.31	0.13	0.08	cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1)
1	0.38	0.45	0.48	N-myristoyltransferase 2 (NMT2)
				Unknown
1	0.45	0.39	0.41	four jointed box 1 (FJX1)
1	0.24	0.18	0.25	leucine rich repeat containing 4 (LRRC4)
1	0.23	0.22	0.07	chromosome 14 open reading frame (C14orf4)
1	0.39	0.37	0.40	downregulated in ovarian cancer 1 (DOC1)
1	0.39	0.41	0.41	zinc finger, CCHC domain containing 2 (ZCCHC2)

claudin-23 (CLDN23). Among them, the reduction of claudin-11 transcript during invasion was confirmed by real-time PCR (Fig. 2A). TJs are intercellular ring structures that seal adjacent cells to one another. The TJ of ECs is mainly responsible for regulating paracellular permeability and maintaining planar cell polarity (221). TJ are composed of transmembrane proteins, which act as a fence to restrict the diffusion of lipids and proteins within the plane of the membrane. These data suggest that breakdown of TJ is associated with, and likely indispensable for changes in cell polarity that occur during EC invasion.

In contrast to TJ components, a series of CAM genes associated with adherens junctions and cell-matrix interactions were upregulated. These include protocadherin10 (PCDH10), protocadherin17 (PCDH17), mucosal addressin cell adhesion molecule-1 (MADCAM1), integrin α2 (ITGA2), integrin αv (ITGAV), and E-selectin (SELE) (Table 1). Protocadherin10 and protocadherin17, cadherin-related receptors belonging to the δ2-protocadherin subfamily, not only exhibit cell-cell adhesion activities but also are believed to possess other functions, such as signal transduction and growth control (222). Our real-time PCR analysis revealed an increase in the expression levels of mRNA for PCDH10 and PCDH17 as ECs undergo invasion, increasing approximately two- and three-fold, respectively (Fig. 2A). Moreover, ITGA2 and ITGAV were induced approximately three-fold at the mRNA level (Table 1), and these data were confirmed by qPCR analysis (Fig. 2A). Another CAM gene with enhanced expression confirmed by real-time PCR analysis is E-selectin (Fig. 2A).

Strikingly, also induced are some transmembrane receptors, which are involved in adhesion in some respects. These induced molecules are CUB domain-containing protein 1 (CDCP1), anthrax toxin receptor 2 (ANTXR2), anthrax toxin receptor 1 (ANTXR1), transcript variant 3, and podocalyxin-like (PODXL) protein. CDCP1, which represents a putative transmembrane protein, containing three CUB domains in its extracellular part, is found to be

A



B

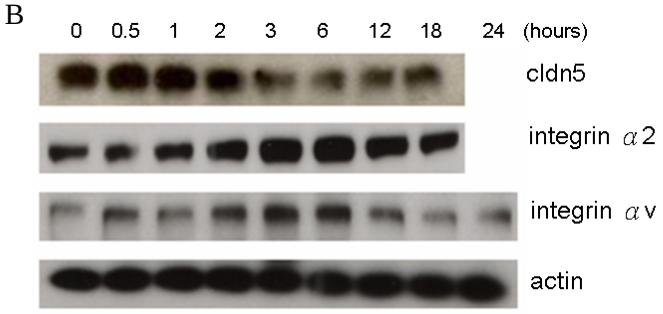


FIGURE 2 **Differential Expression of Cell Adhesion Molecule and Extracellular Matrix Genes during EC Invasion.** (A) Verification of expression profiling at 0, 6, 12, and 18 hours. Invading cells were harvested at each time point and total RNA was purified. The expression of mRNA was assessed by real-time RT-PCR. Data represent means ± SEM ($n \geq 3$) normalized as described in MATERIALS AND METHODS. (*t*-test, *$p<0.05$, **$p<0.01$ compared with time 0) (B) Time course of protein expression for claudin-5, integrin α2, and integrin αv during EC invasion. Invading cells within collagen matrices were collected at indicated times, placed in boiling Laemmli sample buffer, and heated at 95 °C for 10 minutes prior to Western blot analyses using antibodies against indicated proteins. The expression of actin was used as a loading control. Data shown are representative of 3 independent experiments. PODXL, podocalyxin-like protein; CLDN11, claudin-11; PCDH10, protocadherin10; ITGA2, integrin α2; ITGAV, integrin αv; SELE, E-selectin; ANTXR2, anthrax toxin receptor 2; SPP1, secreted phosphoprotein 1; NID2, nidogen 2; NOX4, NADPH oxidase 4.

overexpressed in human colon and lung cancer (223). Intriguingly, upregulation of ANTXR1 and ANTXR2 occurred during EC invasion. ANTXR1 is also known as a tumor-specific endothelial marker (224). ANTXR2 has been reported to bind collagen type IV and laminin directly (225), suggesting an important role for basement membrane matrix assembly. In addition, podocalyxin-like protein is a transmembrane sialomucin that is similar in structure to the well-characterized L-selectin ligand CD34 (226,227). It has been reported to be a novel sinusoidal endothelial cell marker in hepatocellular carcinoma (228,229). The induction of ANTXR2 and PODXL transcripts during invasion was verified by real-time PCR (Fig. 2A). Overall, these findings indicate that augmented expression of these CAM genes is consistent with stimulation by S1P and angiogenic growth factors. These factors would be expected to foster adequate adhesions with adjacent cells and ECM, as well as initiate diverse signaling events that mediate EC invasion.

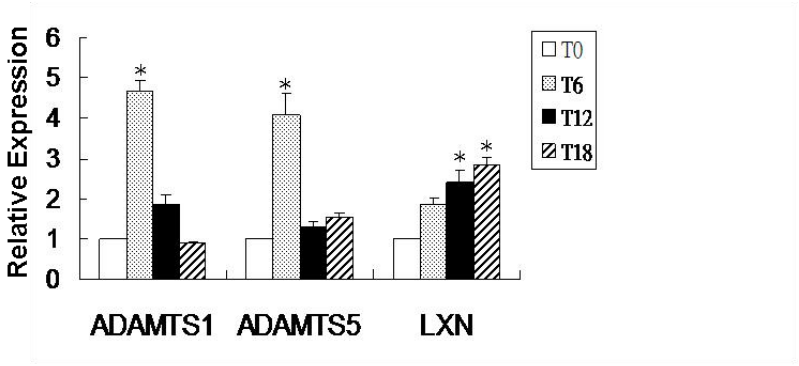
In addition, a series of ECM genes is induced, such as secreted phosphoprotein 1 (SPP1), statherin (STATH), decorin (DCN), transcript variant B, laminin γ2 (LAMC2), collagen, type I, α2 (COL1A2), and nidogen 2 (NID2) (Table 1). The ECM serves as a scaffold in which mechanical forces are established among distal ECs, thereby providing guidance cues in the absence of cell-cell contact (230). Moreover, ECs require adhesions to ECM for migration, invasion, proliferation, and survival, all of which are critical for the process of angiogenesis. We utilized real-time PCR analysis to further confirm the increased expression of mRNA for secreted phosphoprotein 1 and nidogen 2 (Fig. 2A). Secreted phosphoprotein SPP1, also known as osteopontin, is a matricellular protein that is upregulated during vascular injury and wound healing (231,232). Multiple studies have demonstrated upregulation of SPP1 in endometrial tissue where angiogenesis occurs during pregnancy (233-236). Nidogen 2 is a basement membrane-associated molecule that binds collagen I, collagen IV, perlecan and laminin-1

(237,238). Although nidogen-2 is not required for murine basement membrane assembly, dual ablation of nidogen-1 and -2 resulted in embryonic lethal phenotype with microvascular defects (239). Further, in a skin co-culture model, basement membrane and hemidesmosome formation was completely lacking in the absence of both nidogen-1 and -2 (239). Considerable evidence to date demonstrates that nidogen-1 and -2 bind laminin γ1 and γ3 chains. This raises the intriguing possibility that nidogen-2 could interact in this invasion system with laminin γ2, which is also upregulated in a similar pattern. Such induction of diverse ECM components in this system suggests an important role for the ECM as well as basement membrane components to precisely regulate the invasion process.

3.4 Induction of Genes Encoding ECM Proteinases and Their Inhibitors

Cumulative evidence has shown that an intricate balance between ECM proteinases and their inhibitors is critical for mediating diverse physiological events such as lineage decisions during embryogenesis, wound repair, cell migration, vascular stabilization and survival (84-87). Based on distinct domain structures, ECM proteolytic enzymes are divided into several protein families (88), many of which also display an augmented expression in our invasion system (Table 1). Microarray data indicated that A Disintegrin and Metalloproteinase with Thrombospondin-type repeats -1 (ADAMTS1), a secreted metalloproteinase with thrombospondin type I motifs, was induced at the mRNA and protein level during invasion (Fig. 3A, B). As shown in Table 1 and Fig. 3A, another upregulated ADAMTSs is ADAMTS5, which is the major proteinase responsible for cartilage degradation *in vivo* and *in vitro* (240,241). Interestingly, α2-macroglobulin, an endogenous inhibitor of ADAMTS5, was also induced in our microarray analysis (Table 1).

A



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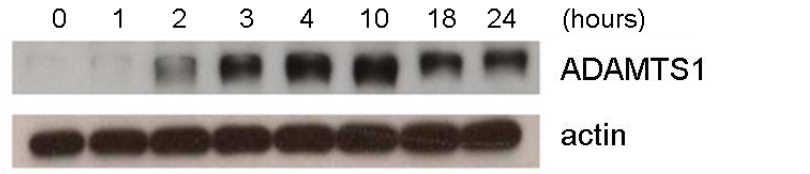

FIGURE 3 **Induction of Selected Genes Encoding ECM Proteases and Protease Inhibitors during EC Invasion.** (A) Verification of mRNA expression for ADAMTS1, ADAMTS5, and LXN observed from microarray analysis. Experiments were performed as described in FIGURE 2. The data represent means ± SEM ($n \geq 3$) (*t*-test, *$p<0.05$ compared with time 0). (B) Time course of protein expression for ADAMTS1 during EC invasion. Protein samples were collected as in FIGURE 2. Antibodies against ADAMTS1 and actin, as a loading control were used in Western blot analyses. Data shown are representative of 3 independent experiments.

Other serine proteases including tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), together with one of their inhibitors, neuroserpin (SERPINI1) were upregulated (Table 1). tPA and uPA are involved in angiogenesis and tumor progression (242-244), whereas neuroserpin is seldom reported to possess endothelial-related functions due to its abundance in nervous system (245). *In vitro* studies with cultured hippocampal neurons demonstrated that neuroserpin mRNA is increased by depolarization with elevated extracellular KCl (246). Interestingly, we have observed an intriguing upregulation of many voltage-gated potassium channels, as well (Table 1). Other upregulated genes encoding ECM proteinases and

protease inhibitors include trypsin 2, matrix metalloproteinase-10 (MMP-10), cathepsin K (CTSK), and latexin (LXN) (Table 1). MMP-10, a soluble proteinase, has been reported to facilitate human capillary tube regression (247,248), and its repression is essential in maintaining vascular integrity (248). Cathepsin K, a cysteine protease that exhibits strong degradative activity against the extracellular matrix, is involved in prostate cancer progression (249). In addition, the increased gene expression of latexin was validated by real-time PCR analysis (Fig. 3A). Latexin, the endogenous protein inhibitor of the A/B subfamily of metallocarboxypeptidases, is discovered as a primary regulator of hematopoietic stem cells (250). These findings suggest that a delicate protease/anti-protease balance orchestrates the process of EC invasion into 3D collagen matrices.

3.5 ADAMTS1 Is Required for EC Invasion in 3-D Collagen Matrices

The data in Figure 3 demonstrate that ADAMTS1 is upregulated at the mRNA and protein level. Because ADAMTS1 is known to associate with the plasma membrane and has been reported to promote collagen degradation (251), we next investigated whether ADAMTS1 is functionally required for EC invasion in collagen matrices. To accomplish this, ECs were treated with small interfering RNA (siRNA) directed to G3PDH, ADAM17, ADAMTS1 and ADAMTS4. Western blot analyses of extracts collected from invading cultures revealed selective knockdown of ADAMTS1 and G3PDH controls with respective siRNAs (Fig. 4A). Photographs (side view) of invading cultures revealed that ECs treated with ADAMTS1 siRNA prior to placing in invasion assays invaded shorter distances and contained larger lumens (Fig. 4B). ECs were tested in invasion assays in the presence and absence of phorbol ester (TPA), which enhanced invasion of ECs placed in 3D matrices (252-254). Quantification of the invasion density revealed decreased numbers of invading cells with ADAMTS1 siRNA treatment (Fig.

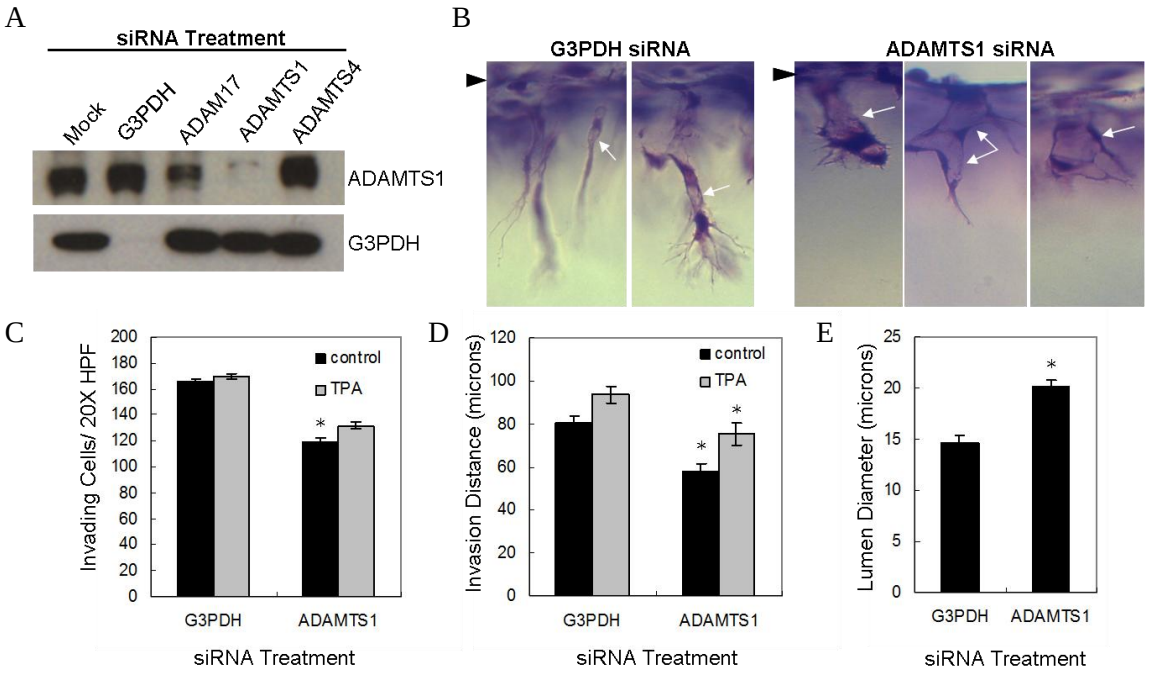

FIGURE 4 **Knockdown of ADAMTS1 Interferes with EC Invasion.** (A) Verification of ADAMTS1 protein suppression. Custom pooled siRNA were delivered to HUVECs prior to seeding on the surface of collagen matrices. Cells were allowed to invade for 24 hours prior to fixing cultures and preparing cell extracts. Antibodies against ADAMTS1 and G3PDH, as an experimental control were used for Western blot analyses. (B) Photographs illustrating the invasion responses (side-view). Cultures were fixed at 24 hour, and stained with toluidine blue. White arrows indicate areas of lumen formation; black arrowheads, monolayer of endothelial cells. (C-E) Quantification of EC invasion responses. Cells were allowed to invade for 24 hours in the absence (control) or presence (TPA) of 50 ng/ml phorbol ester. Cultures were fixed, stained, and quantified for invasion density (C), distance (D), and lumen diameter (E). For invasion density, data represent average numbers of invading cells per standardized field (n=3 fields). For invasion distance and lumen diameter, data from 100 cells were averaged and presented as mean ± SEM (*t*-test, *$p<0.05$).

4C). Invasion distance in ADAMTS1 siRNA-treated ECs was also significantly decreased compared to controls in the presence and absence of phorbol ester (Fig. 4D). Also, quantification of lumen diameter in invading structures revealed ECs treated with ADAMTS1 siRNA assembled into structures containing larger lumens (Fig. 4E). Thus, silencing of ADAMTS1 with siRNA confirms the functional involvement of ADAMTS1 in S1P and growth factor-stimulated EC invasion in 3D collagen matrices.

3.6 Regulated Expression of Genes Implicated in Cell Signaling Pathways

Numerous signaling pathways have been reported to modulate angiogenesis. Here, we demonstrated the differential expression of multiple members of the Wnt and Notch pathways. Multiple Wnt signaling pathway members were identified, including dickkopf homolog 2 (DKK2) and dishevelled associated activator of morphogenesis (DAAM1), which were upregulated (Table 1). In contrast, AXIN1 upregulated 1 (AXUD1) and Frizzled homolog 4 (FZD4) were downregulated (Table 2). These data were confirmed by real-time PCR (Figure 5A). Although the Wnt signaling pathway has a well-defined role in development, its role in angiogenesis and the exact components involved have not been completely delineated. DKK2, unlike DKK1, is a pure inhibitor of the Wnt/β -catenin signaling pathway, and can function either as an agonist or antagonist of Wnt signaling depending on the cellular context (255-257). DAAM1, a member of the mammalian diaphanous-related formins, is shown to communicate with Rho GTPases and Src to regulate the Wnt / planar cell polarity signaling pathway (258). Beyond the transcriptional influences, we showed that Dkk2 and Daam1 proteins were induced as invasion proceeds (Fig. 5D). In addition, consistent with a recent finding that the expression of AXUD1, a downstream target of the Wnt regulator AXIN1, was decreased in lung, kidney, liver and colon cancers (259), AXUD1 is downregulated in our EC invasion system, suggesting

an inhibitory role in vascular morphogenesis. Surprisingly, also downregulated is FZD4, a putative Wnt receptor implicated in retinal angiogenesis (260). These findings raise the possibility that both canonical and non-canonical Wnt pathways may contribute to EC invasion.

Next, a set of genes involved in Notch signaling were downregulated as invasion proceeds. These include delta-like 4 (DLL4), jagged 1 (JAG1), hairy and enhancer of split 1 (HES1), and HES- related with YRPW motif 1 (HEY1) (Table 2). DLL4 and JAG1 are Notch ligands, while HEY1 and HES1, are target genes of Notch. In this experimental system, we confirmed the reduction of DLL4 at the mRNA and protein level (Fig. 5B, D) and a dramatic decrease in HEY1 mRNA by real-time PCR analysis during EC invasion (Fig. 5B). In addition, ligand of numb-protein X (LNX), a RING-type E3 ubiquitin ligase, was found to be upregulated (Table 1). Intriguingly, LNX can cause proteasome-dependent degradation of Notch inhibitor, Numb, thereby enhancing Notch signaling (261).

Additional genes involved in cellular signaling include hedgehog-interacting protein (HIP) and NF-κB inhibitor α (NFKBIa), both of which exhibit downregulation (Table 2, Fig. 5C). Hedgehog interacting protein is a ligand for all three members of the Hedgehog family, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert hedgehog (Dhh). HIP is expressed at high levels in ECs and can antagonize Shh responses in epithelial cells. Its expression is reduced on matrigel during chord formation, as well as in human cancers of the lung, liver and GI tract (262), supporting that by being downregulated. Thus, HIP may function as an angiogenic suppressor, suggesting that activation of the Hedgehog pathway may allow invasion to proceed. In addition, tumor invasion and angiogenesis are associated with NF-κB-mediated gene products, such as matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA), and numerous chemokines (263-265), many of which are also induced during invasion. The signals that activate NF-κB during metastasis or angiogenesis remain incompletely understood,

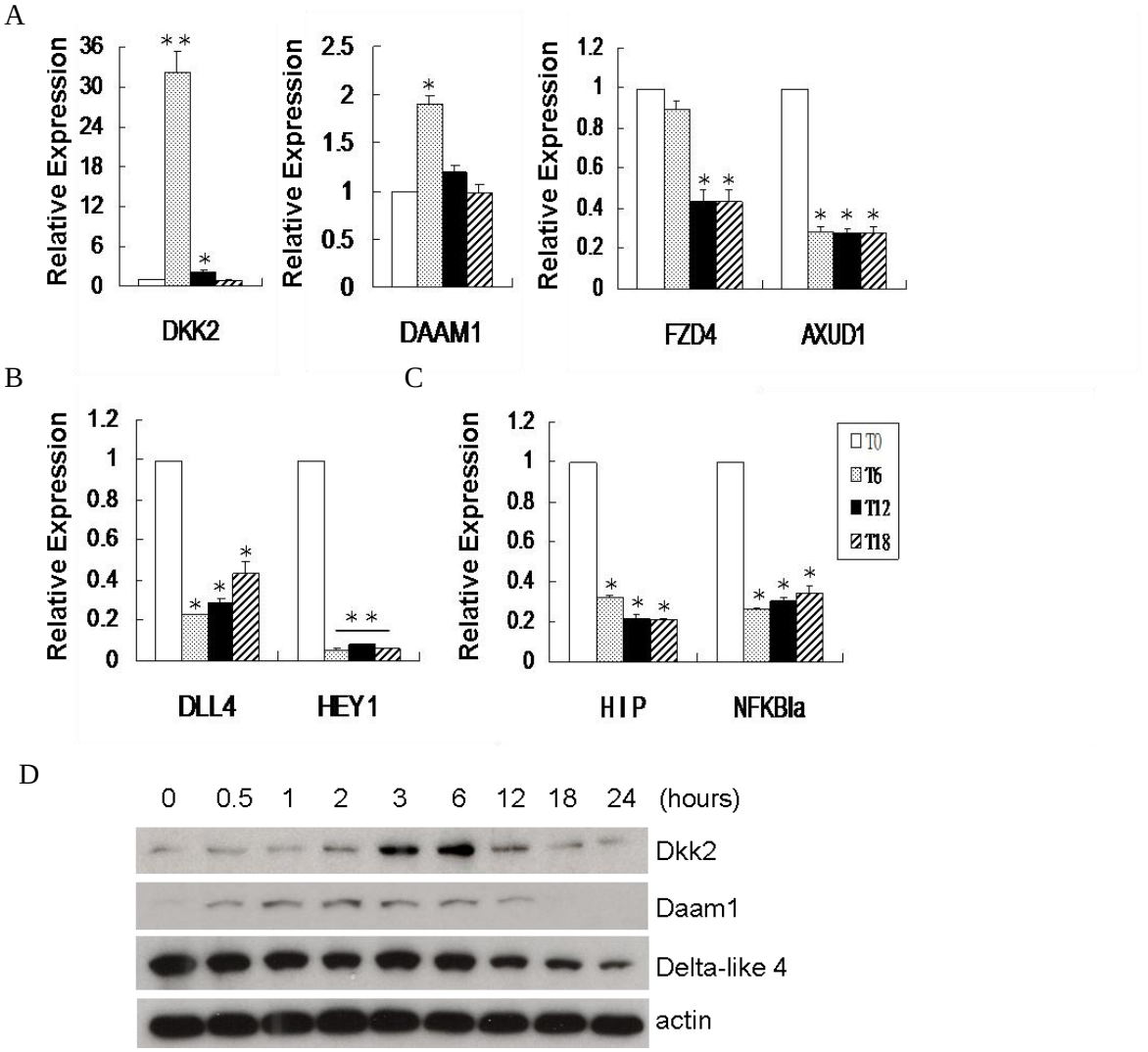

FIGURE 5 **Genes Involved in Various Signaling Pathways Are Regulated during EC Invasion.** Gene expression profiling of members of (A) Wnt, (B) Notch, and (C) hedgehog and NFκB signaling pathways. Experiments were conducted as described in FIGURE 2. Data represent means ± SEM ($n \geq 3$) (*t*-test, *$p<0.05$, **$p<0.01$ compared with time 0). (D) Time course of protein expression for Delta-like 4, Daam1 and Dkk2 during EC invasion. Protein samples were collected as in FIGURE 2. Antibodies against Delta-like 4, Daam1 and Dkk2 and actin, as a loading control were used in Western blot analyses. Data shown are representative of 3 independent experiments. FZD4, frizzled homolog 4; AXUD1, AXIN1 upregulated 1; HEY1, hairy and enhancer of split 1 (HES1)-related with YRPW motif 1; HIP, hedgehog-interacting protein.

but may be related to mutation of NF-κB inhibitor α gene and enhanced degradation of NF-κB inhibitor α protein (266,267). Our findings suggest that a transcriptional regulation of NF-κB inhibitor α may be involved in controlling EC invasion, along with modulation of Wnt, hedgehog, and Notch signaling pathways.

3.7 Notch Signaling Negatively Regulates EC Invasion

Because several members of the Notch pathway were downregulated during EC invasion, we theorized that Notch inhibition would stimulate EC invasion. Treatment with γ-secretase inhibitor, which prevents Notch processing, stimulated EC invasion in a dose-dependent manner (Fig. 6A). To confirm these data, we induced Notch activation in ECs using recombinant lentiviruses to express Dll4 and two constitutively active forms of Notch, Notch1 intracellular domain (N1ICD) and Notch4 intracellular domain (N4ICD) fused to a C-terminal Flag epitope, along with green fluorescent protein (GFP) control. Expression of Dll4 and activated Notch4 markedly blocked the EC invasion in the presence and absence of TPA, whereas activation of Notch1 modestly but significantly reduced invasion (Fig. 6B). In the presence of TPA, the ability of N4ICD and Dll4 to inhibit invasion was less pronounced, but N1ICD, N4ICD, and Dll4 all similarly reduced invasion compared to controls. Moreover, the invading structures formed by activated Notch4- or Dll4-expressing cells were markedly shorter and exhibited less branching than those derived from cells transduced with a GFP expression vector (Fig. 6C, E), suggesting that activation of Notch signaling might alter the decision of EC for invading but also impair the ability to migrate in collagen matrices. We typically achieved lentiviral transduction efficiencies from 50 to 80%. Incorporation of C-terminal FLAG epitopes resulted in an ability to distinguish recombinant from endogenous proteins. Figure 6D shows the expression of FLAG- tagged N1ICD, N4ICD, and Dll4 protein by immunoblotting. It is reported that Notch intracellular

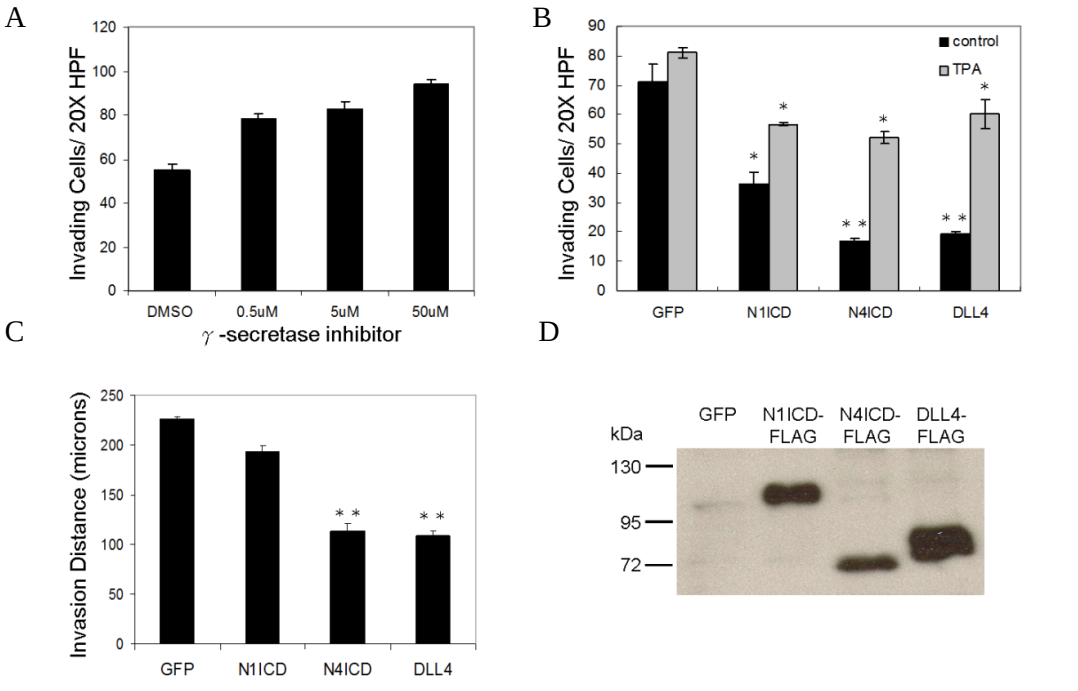

FIGURE 6 **Notch Signaling Negatively Regulates EC Invasion.** (A) Gamma-secretase inhibition promotes EC invasion. Cells were resuspended with indicated concentrations of γ -secretase inhibitor IX or vehicle control (DMSO) for 20 minutes prior to seeding on collagen matrices containing 100 nM S1P for 24 hours. Data represent average numbers of invading cells per standardized field (n=3 fields). (B-C) Activation of Notch signaling inhibits EC invasion. Recombinant lentiviruses were generated that express green fluorescent protein (GFP), Notch1 intracellular domain (N1ICD), Notch4 intracellular domain (N4ICD), and Dll4 fused to a C-terminal FLAG epitope. Three to seven days post-transduction, ECs were allowed to invade for 24 hours with 50 ng/ml phorbol ester (TPA) or without (control), fixed, stained, and quantified for invasion density (n=3 fields) (B), and distance (n=30 cells) (C). (*t*-test, *p<0.05, * *p<0.01). (D) Expression of FLAG-tagged N1ICD, N4ICD, and Dll4 in HUVECs. Cells were treated with TPA and proteasome inhibitor I for 12 hours prior to collecting cell extracts. Western blot analyses were conducted with anti-FLAG monoclonal antibodies. (E) Photographs depicting the inhibition of invasion responses in Notch-activated ECs. From left to right, top-viewed and side-viewed images of 24 hour, toluidine blue-stained cultures are shown in first two columns. Black arrows indicate invading ECs; black arrowheads, EC monolayer. In the third column, localization of N1ICD, N4ICD, and Dll4 proteins was shown by immunofluorescence of top-viewed 12 hour cultures stained with an anti-FLAG monoclonal primary antibody and FITC-conjugated secondary antibodies. The fourth column shows a side view of invading ECs immunofluorescently labeled with FLAG antibodies in 24 hour cultures. White arrowheads indicate monolayer of endothelial cells. Differential interference contrast (DIC) and FITC images were overlayed in the fifth column. Note that N1ICD- and N4ICD-positive cells remain in the monolayer. (F) Localization of FLAG-tagged N1ICD, N4ICD, and Dll4 in HUVECs. ECs were immunofluorescently stained for the Flag epitope. Photographs depicting the nuclear localization of N1ICD and N4ICD are shown in the upper panels. DLL4-Flag immunoreactivity is shown in the lower panels.

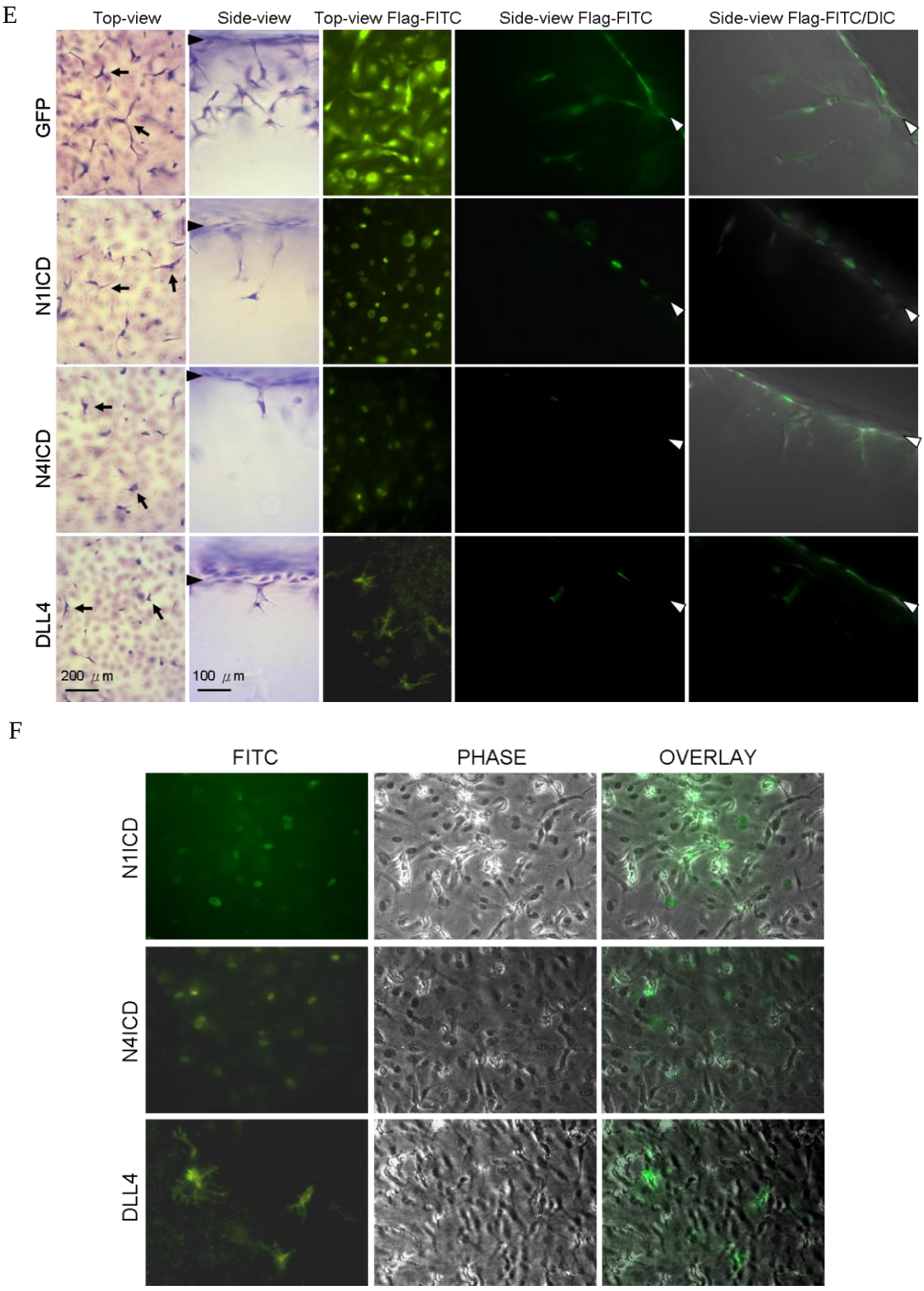

FIGURE 6 **continued.**

domain is targeted for degradation via proteasome pathway (268,269). We have observed similar results in HUVECs, where treatment with proteosome inhibitor stabilized activated Notch protein expression (data not shown). Localization of Notch recombinant proteins within invading ECs was examined by immunofluorescence (Top view Flag-FITC, Fig. 6E, F). Photographs illustrate that GFP distributed throughout the cytoplasm, while N1ICD and N4ICD proteins localized to nucleus, which is typical of constitutively active Notch proteins (270,271), and Dll4 protein localized on the plasma membrane (Fig. 6F). Further, GFP-expressing cells remained in the monolayer and invaded (Fig. 6E, upper right panel). N1ICD- and N4ICD-positive cells, in contrast, did not invade and remained in the monolayer. Dll4-positive cells, like GFP were detectable in invading cells as well as those that remained in the monolayer.

3.8 Identification of Annexin 2 as a Regulator of Endothelial Morphogenesis

In addition, we have undertaken a proteomic screen to identify intracellular targets of S1P that regulate EC invasion and have identified annexin 2 (ANXA2), a Ca^{2+}-regulated membrane binding protein (Fig. 7). Because annexin 2 has been implicated in the formation of new blood vessels (194), we next investigated whether annexin 2 is functionally required for EC invasion in collagen matrices. To accomplish this, we used recombinant lentiviruses that expressed short hairpin RNAs (shRNAs) directed to ANXA2, β2-microglobulin (B2M), and GFP (as a scrambled control) to specifically knock down the expression of annexin 2 or β2-microglobulin and subsequently performed invasion assays. The EC invasion assay utilized here was performed as above. Western blot analyses of extracts collected from invading cultures showed selective knockdown of annexin 2 and β2-microglobulin control with respective shRNAs (Fig. 8B). Photographs of invading cultures illustrated that invasion was significantly decreased in ECs expressing ANXA2 shRNA (Fig. 8A). Quantification of the invasion density revealed decreased

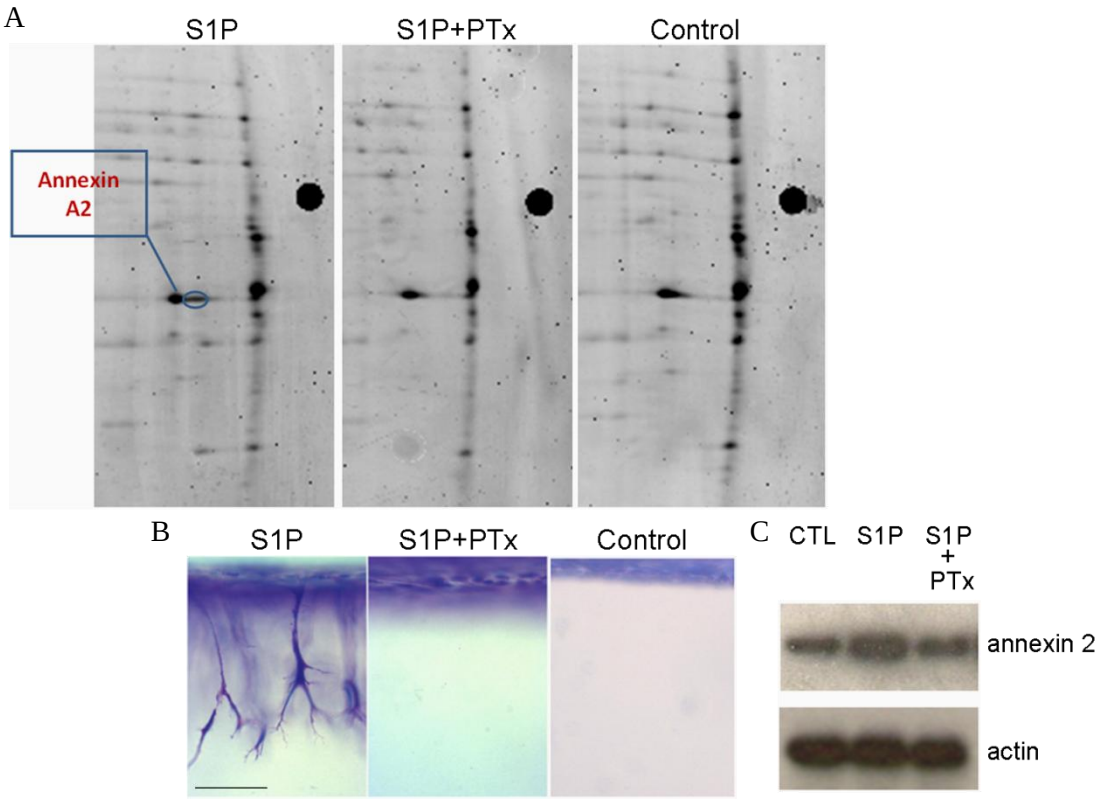

FIGURE 7 **Identification of Annexin 2.** (A) 2D SDS-PAGE analyses of proteins extracted from invasion cultures in the presence of S1P (S1P) or S1P plus pertussis toxin (S1P+PTx), or in the absence of S1P (Control) were performed. The circle highlights the variation that is identified as annexin 2 by mass spectrometry. (B) Photographs depicting invasion responses (side-view). Cultures were fixed at 12.5 hours, stained with toluidine blue and photographed. Scale bar represents 50 μm. (C) Verification of protein expression for annexin 2. Collagen matrices containing invading cells were collected at 12.5 hour, placed in boiling Laemmli sample buffer, and heated at 95 °C for 10 minutes prior to Western blot analyses using antibodies against annexin 2. The expression of actin was used as a loading control.

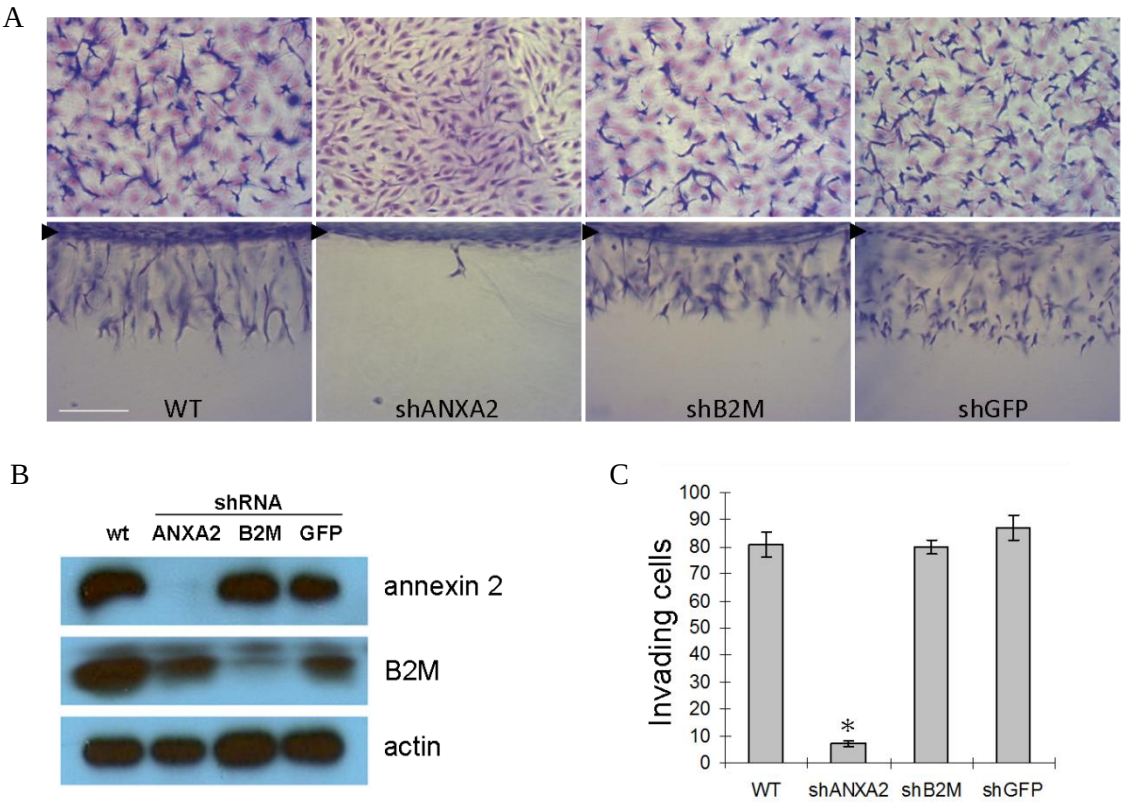

FIGURE 8 **Knockdown of ANXA2 Interfered with EC Invasion.** (A) Photographs illustrating the invasion responses (top-view, upper panel; side-view, lower panel). ECs were transduced with lentiviruses expressing indicated shRNAs and selected with puromycin prior to seeding on the surface of collagen matrices. Cultures were fixed at 24 hours and stained with toluidine blue. Black arrowhead indicates monolayer of endothelial cells. Scale bar, 100 μm. **(**B) Verification of annexin 2 and β2-microglobulin (B2M) protein suppression using extracts collected after 24 hours of invasion. Antibodies against annexin 2, B2M, as an experimental control, and actin, as a loading control were used for Western blot analyses. (C) Quantification of EC invasion density after 24 hours of invasion. Data represent average numbers of invading cells per standardized field (n=3 fields, Students *t*-test, $^{*}p<0.01$).

numbers of invading cells with ANXA2 shRNA treatment (Fig. 8C). Thus, silencing of ANXA2 confirmed a functional involvement of annexin 2 in S1P and growth factor-stimulated EC invasion in 3-D collagen matrices.

3.9 Membrane Translocation of Annexin 2 Was Driven by S1P

Proposed to act as a membrane scaffold protein, most annexin 2 functions are linked to its ability to associate with cellular membranes in a regulated manner (196,272). We, therefore, investigated whether the factors that promote invasion in our assay (e.g. S1P or angiogenic growth factors) stimulate annexin 2 localization from cytosol to plasma membrane. Recombinant lentiviruses that express annexin 2 fused to a C-terminal green fluorescent protein (GFP), referred as to ANXA2-GFP or GFP only were generated and used to examine the distribution of annexin 2 in EC monolayers. ANXA2-GFP has been used successfully to monitor annexin 2 localization in rat basophilic leukemia (RBL) cells and Müller stem cells (198,273). ECs expressing ANXA2-GFP or GFP were treated with S1P or angiogenic growth factors (GF). Fig. 9A shows that ANXA2-GFP was located within the cytoplasm and excluded from the nucleus, while GFP was distributed over both the cytoplasm and nucleus. Treatment with S1P resulted in the apparent enrichment of ANXA2-GFP at the plasma membrane (indicated by arrowheads), whereas GF failed to induce the membrane translocation of ANXA2-GFP and the formation of membrane ruffles. To provide additional biochemical evidence for annexin 2 localization, membranes were prepared from EC monolayers treated with S1P or GF, and then immunoblotted for annexin 2 and various other cytoplasmic and plasma membrane markers. Annexin 2 levels increased in membrane fractions following stimulation with S1P (Fig. 9B), but remained unchanged following stimulation with GF (Fig. 9C). Akt and Erk activation were examined to validate the effects of two stimuli, S1P and GF, respectively. We observed a significant increase

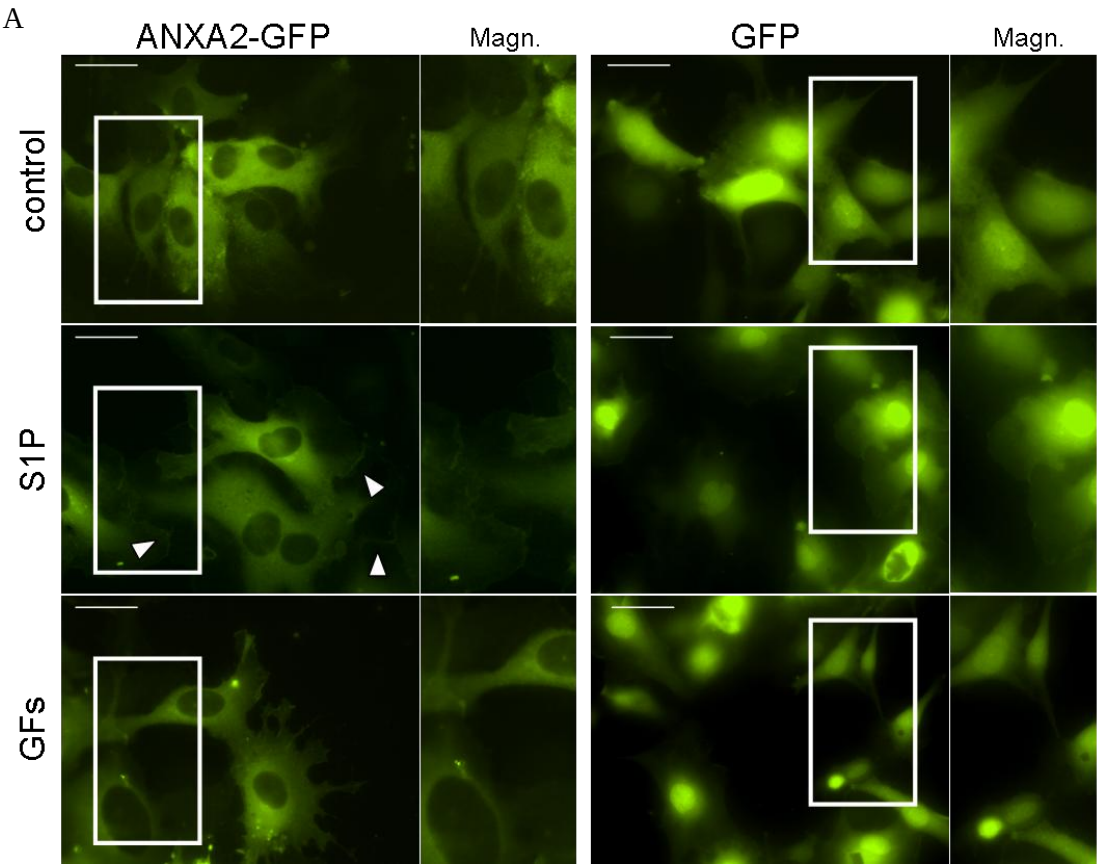

FIGURE 9 **S1P Triggered the Membrane Translocation of Annexin 2.** (A) Fluorescent images of annexin 2 distribution in control, S1P- or GF-stimulated ECs. ECs were transduced with lentiviruses expressing annexin 2 fused to a C-terminal green fluorescent protein (ANXA2-GFP) or green fluorescent protein only (GFP), and selected with blasticidin. Cells that stably express ANXA2-GFP or GFP were treated without (control) or with 1 μM S1P (S1P) or the combination of 40 ng/ml VEGF and bFGF (GF) for 30 minutes prior to fixing and imaging with epi-fluorescent microscopy. The right panels are magnified images of the areas boxed in the left panels (Magn.). Scale bar, 10 μm. (B-C) Biochemical analyses of annexin 2 recruitment to membranes. ECs were stimulated with 1 μM S1P (B) or the combination of 40 ng/ml VEGF and bFGF (GF) (C) for indicated times. Plasma membrane (Membrane) and cytoplasmic (Cytosol) fractions were prepared and immunoblotted with antibodies against indicated proteins. Blots are representative of four independent experiments. Densitometric analyses were quantified by ImageJ software and expressed as means ± SEM in arbitrary units (a.u.) (n=3, Students *t*-test, * $p<0.01$, compared with unstimulated controls).

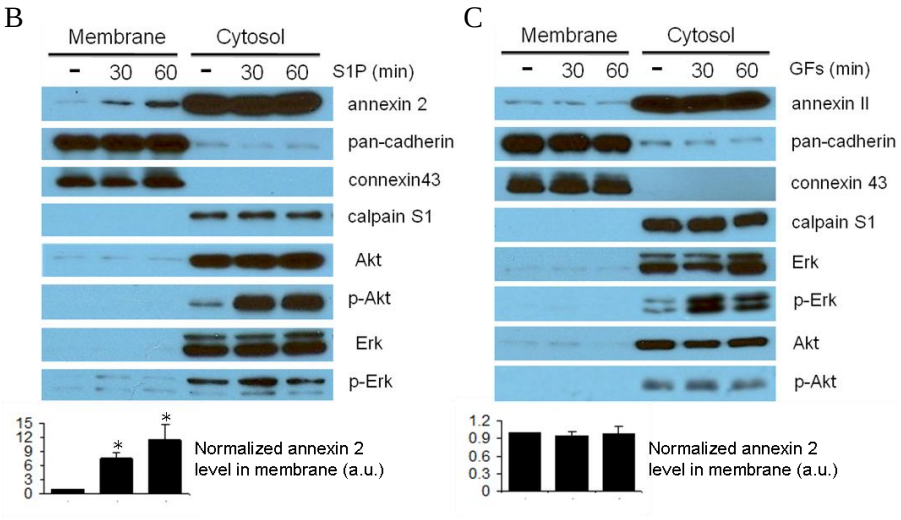

FIGURE 9 **continued.**

in Akt activation with S1P treatment (Fig. 9B) but not with GF treatment (Fig. 9C). In contrast, Erk activation was induced substantially by GF (Fig. 9C) but not S1P (Fig. 9B). Detection of connexin 43 and calpain S1 in membrane and cytoplasmic fractions, respectively revealed successful partitioning of indicated subcellular fractions. Thus, S1P induced membrane translocation of annexin 2 and increased Akt activation.

3.10 Annexin 2 Depletion Attenuated Akt Activation during EC Invasion

EC morphogenesis is regulated by a board spectrum of intracellular signaling pathways, many of which are known to be mediated by S1P receptors (274). To more clearly determine the mechanism by which annexin 2 modulates the EC invasion, we explored whether annexin 2 depletion affected any signaling pathways that are involved in angiogenesis and downstream of S1P receptors, including Akt, Erk and the small GTPases, Cdc42 and Rac1 (Fig. 10). ECs expressing ANXA2 or B2M shRNA were placed in invasion assays on 3-D collagen matrices

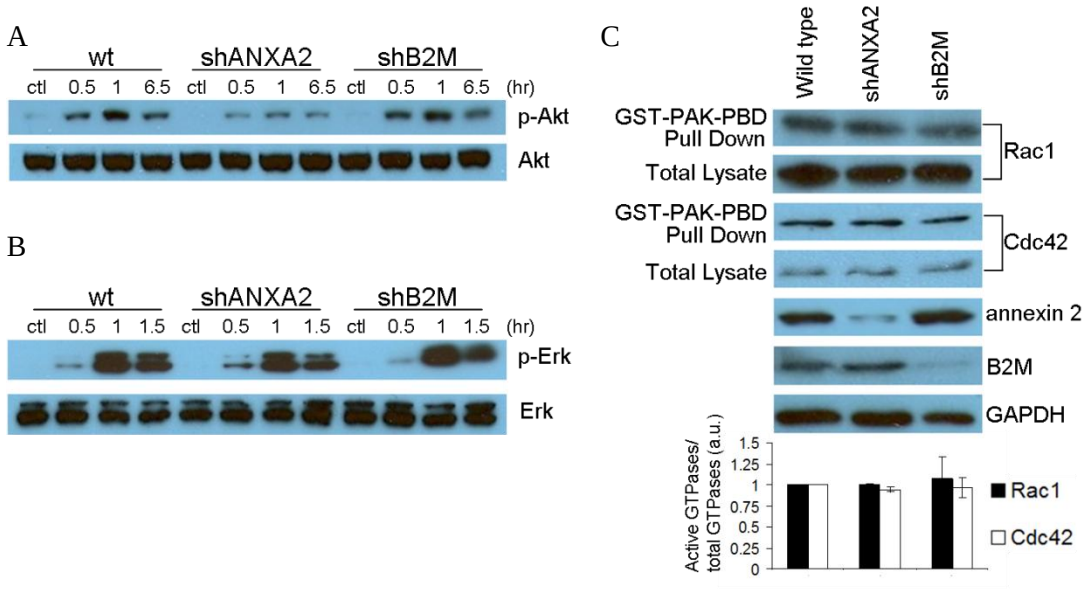

FIGURE 10 **Loss of Annexin 2 Leads to Attenuation of Akt Activation during EC Invasion**. (A-B) Akt and Erk activation in annexin 2- or B2M-depleted cells during EC invasion on 3-D collagen matrices. ECs were transduced without (wild-type, wt) or with lentiviruses producing indicated shRNAs and selected with puromycin prior to seeding on the surface of collagen matrices. Cell extracts were prepared at indicated time-points for Western blot analyses and probed for (A) phosphor-Akt (Ser473) and total Akt, or (B) phosphor-p44/42 MAP kinase (p-Erk) and total Erk. Control (ctl) represents cells cultured on collagen matrices in absence of S1P and growth factors for 0.5 hour. (C) Rac1 and Cdc42 activation is not altered in annexin 2- or B2M-depleted cells during EC invasion. Puromycin-selected ECs were cultured on the surface of collagen matrices for 16 hours. Equal amounts of extracts were prepared and incubated with GST-PAK-PBD protein agarose beads. Eluates and starting lysates were analyzed by Western blot analyses using antibodies against Rac1, Cdc42, annexin 2, or GAPDH (as a loading control). Blots are representatives of three independent experiments. Densitometric analyses represent means ± SEM (n=3).

containing 1 μM S1P and 40 ng/ml GF, and extracts were collected at indicated times for Western blot analyses. Strikingly, silencing of annexin 2 expression decreased Akt activation (Fig. 10A). However, neither annexin 2 nor β2-microglobulin depletion altered Erk activation during EC invasion (Fig. 10B). To examine Cdc42 and Rac1 activation, cell lysates were incubated with GST-PAK-PBD protein agarose beads and analyzed for GTP-bound Cdc42 or Rac1. No apparent differences were observed in the activation of the Rac1 or Cdc42 GTPases following annexin 2 or β2-microglobulin knockdown compared to the non-transduced control in extracts collected at 16 hour of invasion (Fig. 10C). Similar results were seen in extracts analyzed at 3 hour (data not shown). Altogether, annexin 2 does not appear to modulate Erk, Rac1 or Cdc42 activation. Rather, Akt is coupled with an annexin 2-mediated signaling pathway during EC invasion.

3.11 Annexin 2 Modulated Akt Activation by Stabilizing Adherens Junctions

Akt has been demonstrated to serve as a crucial regulator of vascular permeability both *in vivo* and *in vitro* (275-277). In addition, annexin 2 recently was reported to contribute to the establishment of mature endothelial adherens junctions, which are prominent in the maintenance of vascular endothelial integrity (199). Since we observed that membrane translocation of annexin 2 was driven by S1P, which is also known as an endothelial barrier-enhancing agent (41), we determined whether the activation of Akt during EC invasion required the integrity of adherens junctions. Ca^{2+}-dependent, homotypic VE-cadherin interactions were disrupted using three different methods: *1*) addition of increasing concentrations of EGTA to culture medium to chelate extracellular Ca^{2+}, *2*) addition of function blocking antibodies directed to the extracellular domain of VE-cadherin, and *3*) gene silencing with VE-cadherin shRNAs. Cells that underwent various treatments were placed in 3-D invasion assays in the presence of S1P and

GF. All three treatments decreased Akt activation during EC invasion (Fig. 11). Addition of EGTA to culture medium caused attenuated activation of Akt in a dose-dependent manner but did not decrease Erk activation (Fig. 11A). Immunofluorescence analyses revealed that EGTA resulted in a loss of VE-cadherin at intercellular junctions (data not shown). Similar results were obtained by the addition of VE-cadherin blocking antibodies (Fig. 11B) or gene silencing of VE-cadherin (Fig. 11C). Incubation of monoclonal VE-cadherin antibodies have been shown to increase paracellular permeability, inhibit VE-cadherin reorganization, and block angiogenesis *in vitro* (278). VE-cadherin antagonism with CDH5 antibodies resulted in decreased Akt activation during EC invasion. These findings were reproduced using two additional VE-cadherin-specific antisera (BV9, Santa Cruz Biotechnology and ab71285, Abcam; data not shown). Moreover, expression of VE-cadherin shRNA reduced the level of VE-cadherin protein by ~90% and reduced Akt activation, as well (Figure 11C). Gene silencing of platelet endothelial cell adhesion molecule 1 (PECAM1), another transmembrane homotypic adhesion protein expressed on HUVECs, did not alter Akt activation levels. Consistently, Erk activation during EC invasion remained unchanged by either VE-cadherin function blocking antibodies or shRNA expression. These findings demonstrate that disruption of VE-cadherin interactions caused a decrease in Akt activation during EC sprouting in 3-D collagen matrices. This observation is consistent with what we observed following ANXA2 gene knockdown.

Because Akt activation during EC invasion required annexin 2 and intact adherens junctions, we next explored whether annexin 2 regulated endothelial permeability, an endothelium-specific function that is mediated in part by adherens junctions. It has been demonstrated that tyrosine phosphorylation of VE-cadherin is associated with weak junctions and impaired barrier function (180). We observed that phosphorylation of VE-cadherin was higher in annexin 2-depleted cells from invading cultures by Western blot analyses using

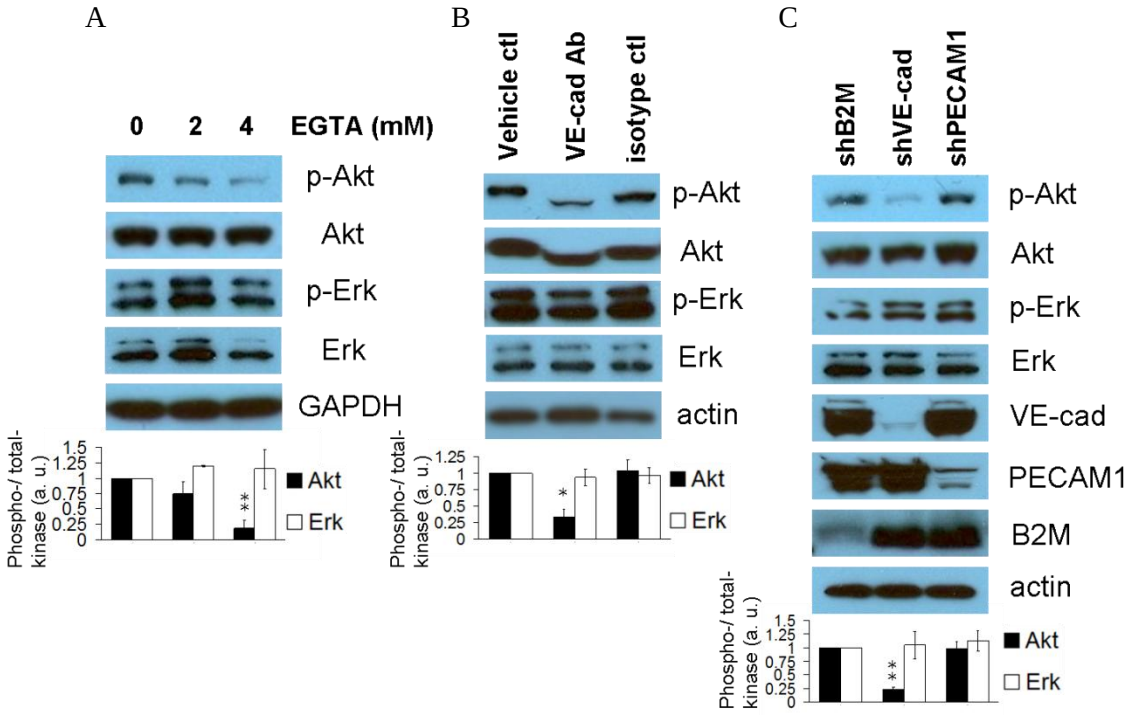

FIGURE 11 **Akt Activation during EC Invasion Requires Intact Adherens Junctions**. (A) The indicated concentrations of EGTA were administered to EC suspensions for 10 minutes prior to seeding on 3-D collagen matrices. Cultures were allowed to invade for 1 hour with the indicated concentrations of EGTA, and extracts were collected for Western blot analyses using antibodies directed to phosphor-Akt (Ser473), Akt, phosphor-p44/42 MAP kinase (p-Erk), Erk or GAPDH (as a loading control). (B) ECs were incubated with 50 μg/ml VE-cadherin antibody (sc-52751, Santa Cruz Biotechnology or 610252, BD Transduction Laboratories), 50 μg/ml isotype control antibody (ab18414, Abcam), or PBS containing 0.1% BSA only (vehicle) for 30 minutes prior to seeding on collagen matrices. Cells were allowed to invade in the presence of indicated antibodies for 1 hour, and extracts were prepared and immunoblotted for phosphor-Akt (Ser473), Akt, phosphor-p44/42 MAP kinase (p-Erk), Erk or actin (as a loading control). (C) ECs were transduced with lentiviruses expressing indicated shRNAs for 3 days and subsequently used for invasion assays. Extracts were collected following 1 hour of invasion and analyzed by Western blot using the indicated antibodies. Blots are representatives of three independent experiments. Densitometric analyses represent means ± SEM (n=3, Students *t*-test, $^{*}p<0.05$, $^{**}p<0.01$, compared with corresponding controls).

antibodies directed against phospho-tyrosine 731 of VE-cadherin (Fig. 12A). These data suggest that annexin 2 may positively regulate junctional integrity, thereby enhancing barrier function. We next determined whether annexin 2 contributed to regulation of endothelial permeability. To investigate this, the transendothelial electrical resistance (TEER) of annexin 2-depleted cells was assessed. Cells were transduced with shB2M, shANXA2, and shVE-cad lentiviruses and seeded on Transwell inserts. The TEER value was monitored 24, 48 and 72 hours post-shRNA administration. A decrease in TEER of annexin 2-depleted cells was observed 48 hours post-transduction (-18.37%, p=0.038, at 48 hours and -36.2%, p=0.007, at 72 hours, compared with B2M control at the same time point, Fig. 12B). Specific downregulation of VE-cadherin also caused a significant decrease in TEER (-48.15%, p=0.006, at 48 hour and -53.15%, p=0.002, at 72 hour, Fig. 12B) that remained stable between 48 and 72 hours. Following quantification, successful knockdown of annexin 2, VE-cadherin and B2M was verified by Western blot analyses using cell extracts collected from cultures (data not shown). We further analyzed the effect of annexin 2 on the endothelial barrier stabilization in the presence or absence of S1P using FITC-dextran permeability assays. In these experiments, ECs were cultured on Transwell inserts and transduced with lentiviruses expressing shRNAs specific to ANXA2, B2M, or VE-cadherin. When cells reached confluence, FITC-dextran was added to the upper chamber and diffused at a measurable rate across the EC monolayer. Quantification of FITC-dextran levels in bottom wells is shown in Fig. 12C. Knockdown of annexin 2 significantly increased endothelial permeability compared to B2M controls in both conditions (+14.17%, p=0.036, without S1P treatment; +29.23%, p=0.012, with S1P treatment). Intriguingly, S1P treatment was less effective at decreasing permeability (i.e. enhancing barrier function) in annexin 2-depleted cells compared to B2M-depleted cells. S1P only effectively enhanced the barrier stabilization of B2M-depleted cells (-23%, p=0.006) but not annexin 2-depleted cells (-12%, p=0.143). This

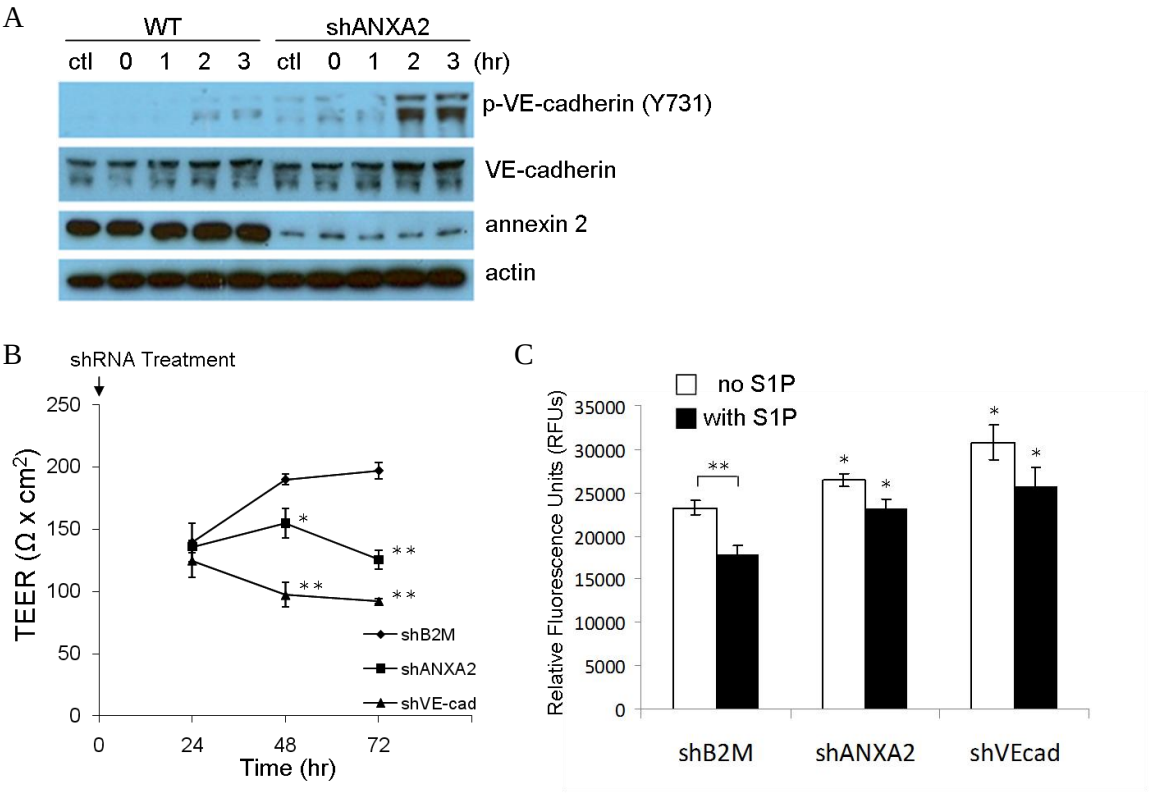

FIGURE 12 **Annexin 2 is Functionally Required for the Junctional Integrity of EC Monolayers.** (A) Knockdown of annexin 2 augments tyrosine phosphorylation of VE-cadherin during EC invasion. ECs treated with (shANXA2) or without (wild-type, WT) lentiviruses that express ANXA2 shRNA were used for invasion assays. Extracts were prepared from invading cultures at indicated time-points and immunoblotted with phosphor-VE-cadherin (Y731), VE-cadherin, annexin 2, or actin (as a loading control). Control (ctl) represents ECs that were cultured on collagen matrices in the absence of S1P and growth factors for 0.5 hour. (B) Transendothelial electrical resistance (TEER) in annexin 2-depleted EC monolayer. ECs were seeded on Transwell inserts and subsequently transduced with lentiviruses expressing indicated shRNAs. The resistance was monitored at the time-points indicated after lentiviral transduction. Data were normalized and presented as average TEER values ± SEM from two experiments (n=3, Students *t*-test, $^{*}p<0.05$, $^{**}p<0.01$, compared with B2M controls at the same time-point). (C) FITC-dextran flux permeability assay. ECs seeded on Transwell inserts were transduced with lentiviruses expressing shRNAs indicated and grown to confluence. EC monolayers were serum-starved for 6 hours and treated with or without 1 μM S1P for 1 hour prior to adding FITC-labeled dextran into the upper chambers. Endothelial permeability (fluorescence in the lower chamber) was measured at 1 hour after the addition of FITC-dextran. Data presented are average values ± SEM from representative experiments (n=4, Students *t*-test, $^{*}p<0.05$, $^{**}p<0.01$, compared with B2M controls).

result revealed that ECs depleted of annexin 2 are less responsive to S1P, suggesting a role for annexin 2 in S1P-enhanced endothelial barrier function. Similarly, expression of VE-cadherin shRNA augmented the flux across EC junctions compared to B2M controls either with (+43.61%, p=0.037) or without S1P treatment (+32.5%, p=0.031) as expected. S1P did not significantly strengthen the barrier function of VE-cadherin-depleted cells (-16.35%, p= 0.097).

It has been shown that blocking adherens junctions interferes the correct organization of tight junctions or the expression of tight junction components in various cell systems (192,279,280). We, therefore, further examined whether annexin 2 depletion altered the organization or expression of claudin-5, an EC-specific tight junction protein (171). However, neither expression nor localization of claudin-5 was altered by appreciable knockdown of annexin 2 or VE-cadherin (Fig. 13). These results indicate that annexin 2 is functionally involved in regulating the integrity of adherens junctions.

3.12 S1P Stimulation Induced the Association of Annexin 2 with VE-Cadherin

The above results show that annexin 2 stabilized adherens junctions, and S1P stimulated membrane recruitment of annexin 2. We reasoned, therefore, that S1P might promote the association of annexin 2 and VE-cadherin. To test this, we isolated membrane fractions of S1P- or angiogenic growth factors (GF)-stimulated EC monolayers and performed immunoprecipitations using an antibody directed to VE-cadherin. Eluates were probed for annexin 2, VE-cadherin and proteins known to form a complex at cell-cell contacts (β-catenin and actin) (172). As shown in Fig. 14A, S1P (but not GF) stimulation increased the amount of annexin 2 that immunoprecipitated with VE-cadherin, while equivalent quantities of VE-cadherin were immunoprecipitated in all conditions. Moreover, this increase appeared independent of the association of VE-cadherin with β-catenin and actin. In addition, we

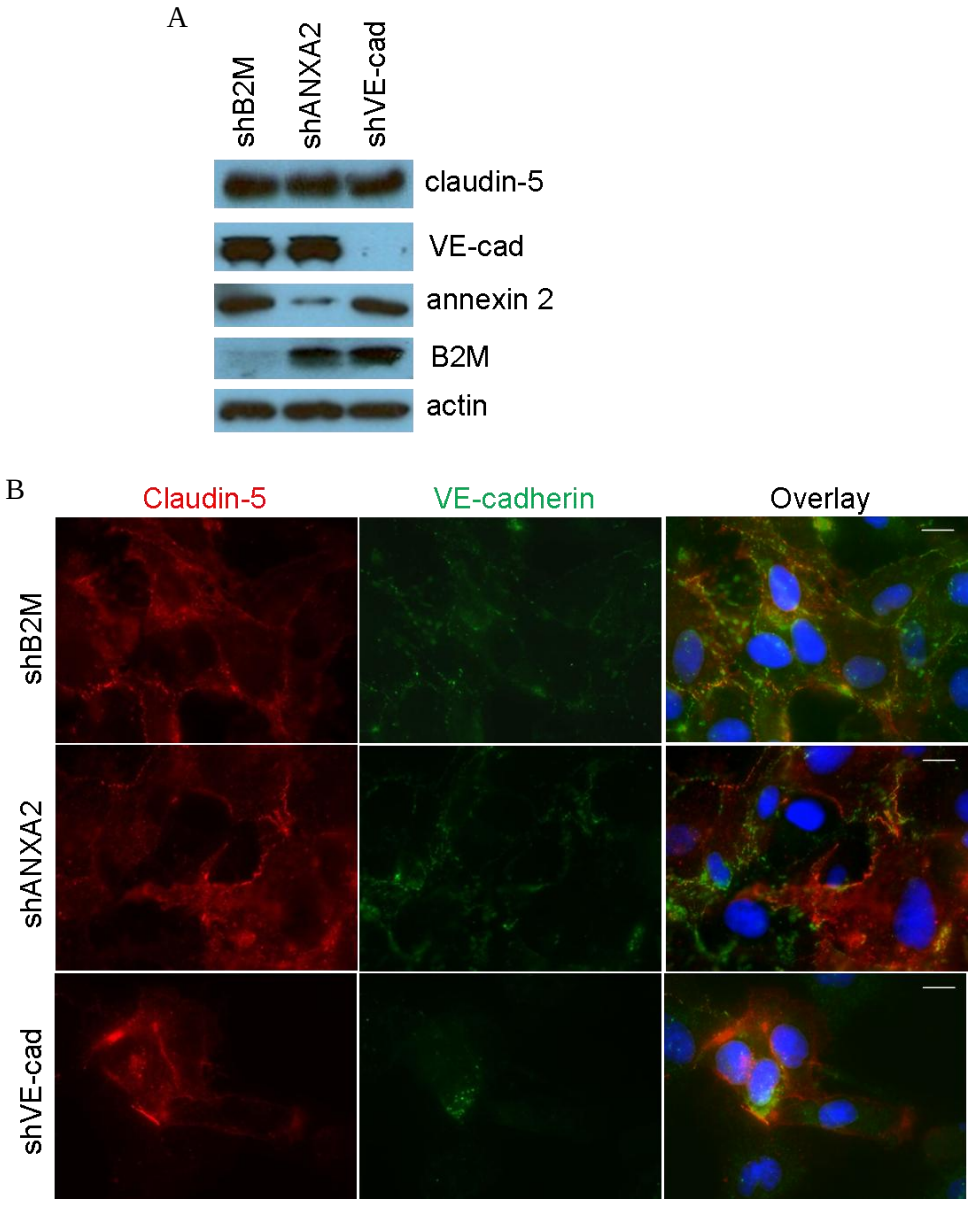

FIGURE 13 **Expression and Localization of Claudin-5 in Annexin 2-Depleted HUVECs.** (A) Western blot analyses of claudin-5 expression in extracts of B2M-, annexin 2-, VE-cadherin-depleted HUVECs in confluent conditions. HUVECs were transduced with lentiviruses expressing indicated shRNAs for 3 days and extracts were collected for western blot analyses. (B) Immunofluorescence microscopy of B2M-, annexin 2-, VE-cadherin-depleted HUVECs. ECs were transduced with lentiviruses expressing indicated shRNAs for 3 days and reseeded on glass coverslips for culture overnight prior to fixing and double stained with anti-claudin-5 (red) and anti-VE-cadherin (green). Images were analyzed using epi-fluorescence microscopy. Scale bar, 5 μm.

performed the reverse immunoprecipitation experiment. Extracts of S1P-treated ECs expressing annexin 2 tagged with a C-terminal FLAG epitope (ANXA2-FLAG) were incubated with an antibody against FLAG to verify the physical interaction of annexin 2 with VE-cadherin, while N-cadherin was not detected in anti-annexin2-FLAG IPs (Fig. 14B).

To demonstrate the co-localization of annexin 2 and VE-cadherin following S1P stimulation, immunofluorescence analyses of VE-cadherin were performed using ECs expressing ANXA2-GFP in the absence or presence of S1P. In the absence of S1P, little annexin 2 was detectable at cell-cell junctions (Fig. 14C). However, ECs stimulated with S1P showed increased colocalization of annexin 2 and VE-cadherin at cell-cell contacts in the presence of S1P. Together, the results indicate that S1P induced annexin 2 translocation to the plasma membrane, where annexin 2 complexed with VE-cadherin.

3.13 Akt Activation Restored Annexin 2-Mediated Aberration of EC Invasion

We next sought to determine whether annexin 2-mediated attenuation of Akt activation is a crucial event in EC morphogenesis and responsible for impaired invasion responses. Treatment of ECs with a specific Akt inhibitor (Akt inhibitor X) which selectively inhibits the phosphorylation of Akt and its *in vitro* kinase activity (281), resulted in a dose-dependent decrease in Akt activation and invasion responses (data not shown). To analyze the potential involvement of Akt in annexin 2-mediated EC morphogenesis, we introduced a constitutively active version of Akt (myr-Akt) into annexin 2-depleted ECs. Recombinant lentiviruses that express myr-Akt or GFP were generated and used to induce Akt activation in ECs stably expressing shRNAs against ANXA2 or B2M. Western blot analyses of extracts collected from invading cultures revealed successful protein expression of myr-Akt and GFP as well as selective knockdown of annexin 2 and B2M control (Fig. 15B). Imaging of invading cultures

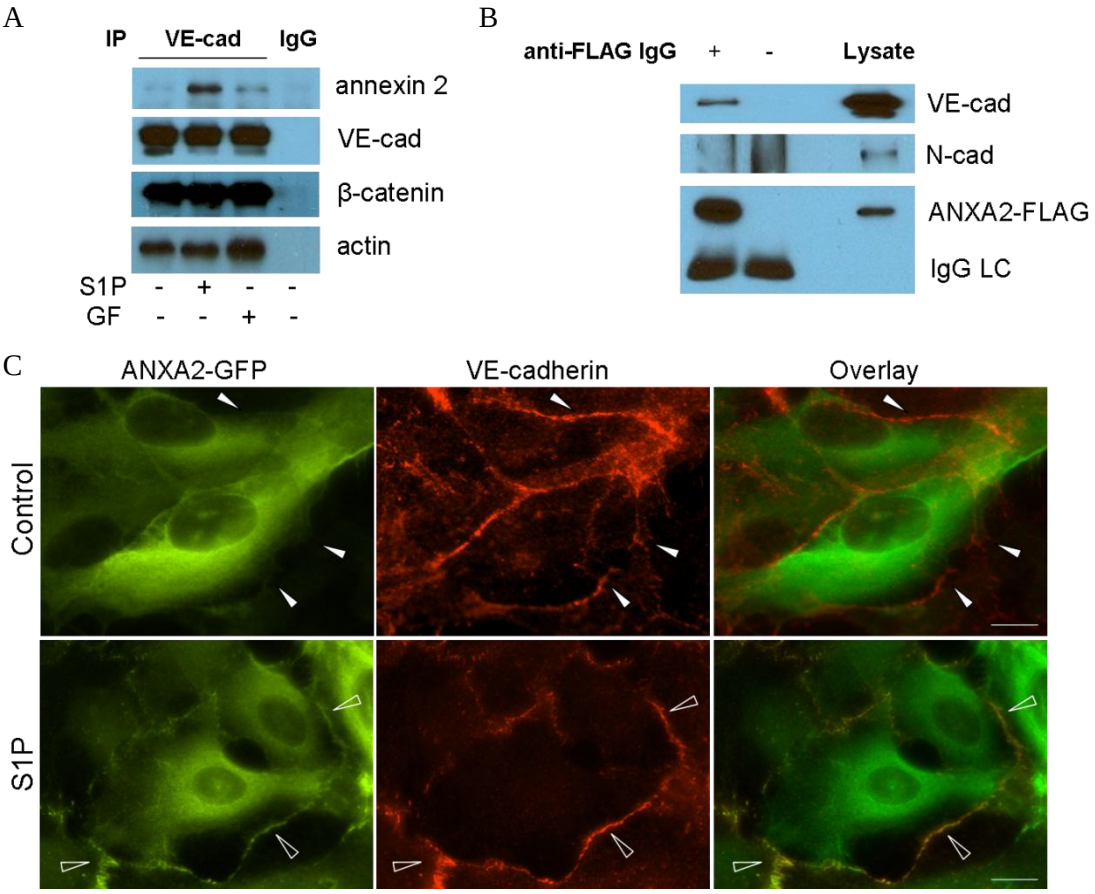

FIGURE 14 **S1P Induced Annexin 2 Coupling with VE-Cadherin**. (A) ECs were serum-starved for 4 hours prior to stimulation without or with 1 μM S1P or the combination of 40 ng/ml VEGF and bFGF (GF) for 1 hour. Membranes were prepared and immunoprecipitated with a polyclonal VE-cadherin antibody or isotype control. The immunoprecipitates were processed for Western blot analyses and probed for annexin 2, VE-cadherin, β-catenin and actin. (B) ECs that express annexin 2 tagged with a C-terminal FLAG (ANXA2-FLAG) were serum-starved and treated with 1 μM S1P for 1 hour. Immunoprecipitations were performed on cell lysates using a polyclonal FLAG antibody or isotype control, and immunoprecipitates were analyzed by Western blot, probed for VE-cadherin, N-cadherin, and FLAG. (C) ECs seeded on glass coverslips were serum-starved, treated with or without (Control) 1 μM S1P for 1 hour prior to fixing and stained with anti-VE-cadherin monoclonal antibody and Alexa Fluor 594 goat anti-mouse IgG. Images were analyzed using epi-fluorescence microscopy. Solid arrowheads point to junctions with missing annexin 2 signals. Open arrowheads indicate annexin 2 and VE-cadherin co-localization. Scale bar, 5 μm.

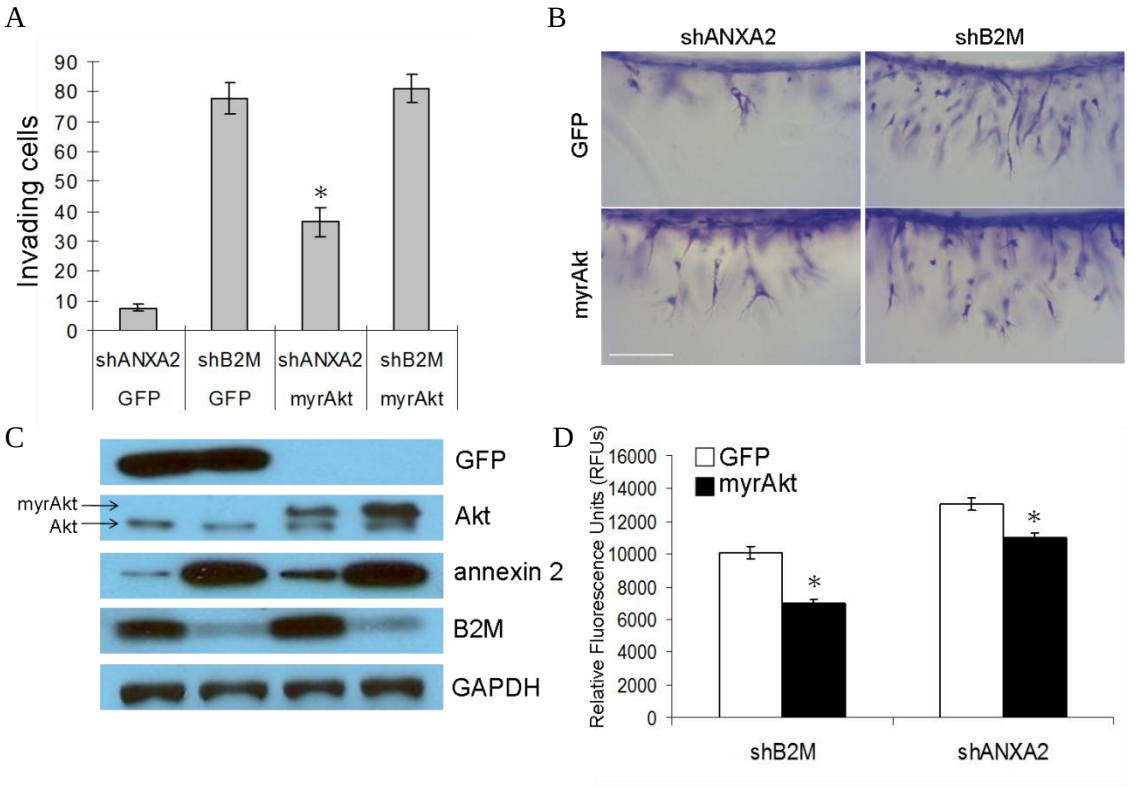

FIGURE 15 **Akt Activation Restored EC Invasion and Endothelial Barrier Integrity Induced by ANXA2 Silencing**. (A) Quantification of EC invasion density. ECs were transduced with lentiviruses expressing indicated shRNAs and selected with puromycin. Cells stably producing shRNAs against ANXA2 or B2M were subsequently transduced with recombinant lentiviruses that express myr-Akt or GFP. Cultures were allowed to invade for 24 hours, fixed, stained, and quantified. Data represent average numbers of invading cells per standardized field (n=3 fields, Students *t*-test, $^{*}p<0.05$, versus cells depleted of annexin 2 but expressing GFP). (B) Verification of gene suppression (ANXA2 and B2M) and expression (myr-Akt and GFP). Cells were allowed to invade for 24 hours prior to preparing cell extracts. Western blot analyses were performed using antibodies as indicated. (C) Photographs depicting the invasion responses (side-view). Cultures were fixed at 24 hours, stained with toluidine blue, and photographed. Scale bar, 100 μm. (D) FITC-dextran flux permeability assay. ECs were transduced with recombinant lentiviruses that express myr-Akt or GFP for 3 days. Subsequently, cells were seeded on Transwell inserts, transduced with lentiviruses expressing shRNAs indicated and grown to confluence. EC monolayers were serum-starved for 6 hours and treated with 1 μM S1P for 1 hour prior to adding FITC-labeled dextran into the upper chambers. Endothelial permeability (fluorescence in the lower chamber) was measured 1 hour after the addition of FITC-dextran. Data presented are average values ± SEM from three experiments (n=4, Students *t*-test, $^{*}p<0.05$, compared with GFP controls).

revealed that the invasion density and length were both retrieved in annexin 2-depleted ECs that expressed Myr-Akt (Fig. 15C). Quantification of the invasion density showed that shB2M and GFP co-expressing cells were not hindered in their ability to invade. However, knockdown of annexin 2 (shANXA2) was not rescued by GFP (Fig. 15A). Knockdown of annexin 2 (shANXA2) combined with GFP expression was comparable to ANXA2 silencing alone (Fig. 8C). However, myr-Akt expression partially restored the ability of annexin 2-depleted cells to invade while it did not enhance invasion in ECs depleted of B2M. Moreover, since specific VE-cadherin reduction diminished Akt activation during EC invasion (Fig. 11C), we also determined whether activated Akt could overcome deficiencies of VE-cadherin expression and rescue invasion responses. Examination of invasion cultures using ECs expressing either VE-cadherin or B2M shRNA revealed that myr-Akt recovered VE-cadherin-mediated impairment of EC invasion as well (Supplemental Fig. 5). Besides, myr-Akt also enhanced the endothelial barrier integrity in ECs depleted of B2M, annexin 2, or VE-cadherin in the presence of S1P (Fig. 7D, and Supplemental Fig. 5C). These results indicate that Akt is activated downstream of an adherens junction-mediated pathway and Akt activation requires annexin 2 during endothelial morphogenesis.

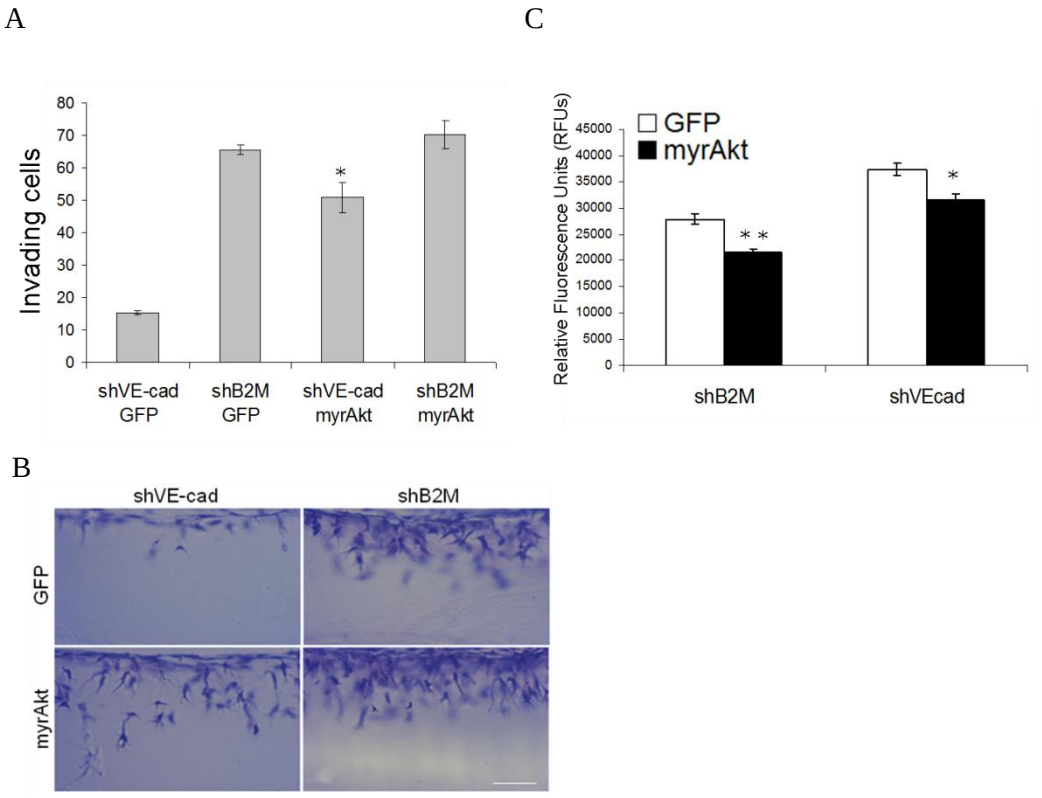

FIGURE 16 **Reintroducing Constitutively Active Akt Compensates for VE-Cadherin Depletion and Rescues EC Invasion.** (A) Quantification of EC invasion density. ECs were transduced with lentiviruses producing indicated shRNAs for 3 days, and subsequently administered lentiviruses expressing myr-Akt or GFP for an additional three days. Cultures were allowed to invade for 24 hours, fixed, stained, and quantified. Data represent average numbers of invading cells per standardized field (n=3 fields, Students *t*-test, $*p<0.05$, versus that of cells depleted of VE-cadherin but expressing GFP). (B) Photographs depicting invasion responses (side-view). Cultures were fixed at 24 hours, stained with toluidine blue and photographed. Scale bar represents 100 μm. (C) FITC-dextran flux permeability assay. ECs were transduced with recombinant lentiviruses that express myr-Akt or GFP for 3 days. Subsequently, cells were seeded on Transwell inserts, transduced with lentiviruses expressing shRNAs indicated and grown to confluence. EC monolayers were serum-starved for 6 hours and treated with 1 μM S1P for 1 hour prior to adding FITC-labeled dextran into the upper chambers. Endothelial permeability (fluorescence in the lower chamber) was measured at 1 hour after the addition of FITC-dextran. Data presented are average values ± SEM from three experiments (n=4, Students *t*-test, $^{*}p<0.05$, $^{**}p<0.01$, compared with GFP controls).

4. DISCUSSION*

A variety of studies have utilized genome-wide screening and differential gene analysis to identify regulated genes in 3-D models of *in vitro* angiogenesis (225,282-284). In spite of minor variations in the experimental conditions and the way ECs are seeded, a high degree of consistency emerges from these data, and numerous findings are in accordance with *in vivo* angiogenic events. In this study, we have examined the gene expression profile of primary EC invasion stimulated by angiogenic growth factors and S1P.

The three-dimensional model utilized here incorporates the lysosphingolipid, S1P, which is present in serum and released by activated platelets. To our knowledge, this is the first study to investigate S1P- and growth factor-induced gene expression changes in primary endothelial cells. The assay system used here mimics a wound environment where S1P release would be coupled with local production of VEGF and bFGF to stimulate granulation tissue formation and new blood vessel growth. The combination of these factors not only stimulates EC invasion, but likewise upregulates endothelial expression of the surface markers E-selectin and mucosal addressin cell adhesion molecule-1 (MADCAM1) that are involved in directing leukocyte extravasation (285,286). Interestingly, NADPH oxidase 4 and cytochrome p450 are upregulated significantly at 6 hours of invasion (Table 1, Fig. 2A), which suggests this pathway may be initiated to activate the endothelium following exposure to S1P and angiogenic growth factors.

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Cytochrome p450 monooxygenases and NADPH oxidase can induce E-selectin, ICAM-1, and VCAM-1, whereas MADCAM1 induction may depend exclusively on CYP450-derived oxidants (287). Further, multiple chemokines and chemokine receptor mRNA expression were observed to be significantly regulated with time. These are CCL1, CXCR4 and CCRL1. Other relevant molecules include THY1, TNFSF11, TNFSF18, and COX-1. A recent study has shown that stable knock-down of one S1P receptor, $S1P_1$ in ECs decreased the expression of several CAMs and influenced the inflammatory response of ECs (288). These data suggest that exposing the endothelium to S1P and angiogenic growth factors not only stimulates new blood vessel growth, but also alters EC expression of several inflammatory proteins that promote leukocyte extravasation and wound healing. These data are consistent with the well-known link between inflammatory cells and angiogenesis not only during tissue healing, but in many other contexts, as well, including pregnancy (289-291), choroidal neovascularization (292-296) and tumor angiogenesis (297-299). These data are consistent with the recognized role for S1P as an important inflammatory mediator that regulates lymphocyte egress, graft rejection and recurrent multiple sclerosis (300-303).

Cell-cell and cell-matrix contacts are integral in regulating angiogenesis. We have observed the downregulation of a set of genes associated with tight junctions, including CX40, CX45, ZO2, CLDN5, CLDN11, and CLDN23 (Table 1, Fig. 2). Connexins (CXs) have been recognized as tumor suppressors, and whose expression is relevant to tumor progression from diverse origins, such as breast, lung, skin, liver, bladder, esophagus, and prostate cancer (304). Among members of connexin family, connexin 26 and 43 are most extensively studied and found to be downregulated in various cancers and tumor angiogenesis (304). In a recent study, decreased expression of connexin 40 is detected in conditional PTEN knockout mice, which display enhanced tumorigenesis (305). Similarly, alterations in the expression levels of claudins

(CLDNs) occur in various cancers. A thorough survey of CLDN expression in normal and neoplastic tissues indicates that the expression of most CLDN genes appears decreased in cancers, while CLDN3, CLDN4, and CLDN7 are elevated in many malignancies (306). These data suggest that the deprivation of endothelial tight junction is a prerequisite for the onset of angiogenesis. In addition, our finding regarding the increased expression of integrin α2 and αv fits the well-defined roles for integrin receptors in sprouting angiogenesis. These integrins not only provide the adequate interactions with ECM but also emit intracellular signals to control cell shape and contractility. Integrin α2β1 modulates VEGF-induced angiogenesis *in vivo* by a notable induction of actin polymerization through inhibition of PKA (cAMP-dependent protein kinase A) activity (62,65,307). Like α2β1 in collagen matrices, αvβ3 in combination with α5β1 control EC shape changes, including vacuolation and lumen formation in fibrin matrices (66). Therefore, it seems plausible that multiple integrins may act together to create combinations of signals for neovascularization. Another intriguing finding in this study is the induction of two members of δ2-protocadherin gene family, PCDH10 and PCDH17 (Fig. 2). So far, little is known about the expression and function of these two protocadherins. However, it is clear that protocadherins may be more involved in intracellular signaling than in adhesive functions. One member of δ2-protocadherin subfamily, PCDH8/PAPC, is found to interact with the Frizzled 7 receptor, and can regulate the activity of the small GTPase, Rho and JNK, indicating a role in modulating the Wnt/PCP signaling pathway (308,309). Perturbing PCDH8 activity in mice leads to the disruption of somite epithelialization during development (310), yet mice defective in PCDH8 not only possess normal skeleton but also are viable and fertile (311). These results suggest that functional redundancy among protocadherins exists.

ECM remodeling is crucial for all aspects of vascular biology and tumor progression. The ECM can be remodeled by biosynthesis and proteolytic degradation. We have demonstrated that

the expression of several ECM genes is regulated during EC invasion (Table 1, Fig. 2). Many ECM components identified in our microarray analysis were likewise upregulated in other studies using 3D models of *in vitro* angiogenesis (225,282) and proven as regulators of angiogenesis or vascular regression events (230,312). Strikingly, decorin, a proteoglycan that plays an important role in regulating collagen fibril organization (313) was induced in our invasion system (Table 1). Whereas decorin can inhibit EC migration and tube formation (314,315), a line of evidence showed that decorin promotes endothelial tube formation in collagen gels (316). These conflicting results may be due to the complexity of ECM.

In addition to matrix molecules, a set of genes associated with the proteolytic processes of ECM was upregulated, including ADAMTS1, ADAMTS5, A2M, tPA, uPA, SERPINI1, MMP-10, CTSK, PRSS2, and LXN (Table 1, Fig. 3). These molecules function as proteases and endogenous protease inhibitors. Cumulative evidence implies that the balance between proteases and their inhibitors is critical for angiogenesis. Most of these proteases are expressed in the extracellular milieu as inactive forms, which can be activated upon proteolytic cleavage by several families of proteases. To date, we have become aware that those proteases can target many non-ECM proteins, including growth factors, growth factor receptors, cell-associated molecules, and cytokines. Because several clinical trials using ECM proteases and their inhibitors as targets unfortunately appear inefficacious (317-319), a full understanding of the involvement and function of these molecules will be helpful for future treatments.

Gene expression profiling of the 3-D cultures revealed that S1P and angiogenic growth factor stimulation upregulated ADAMTS1, whose expression has been correlated with the highly vascularized ovulation cone in ovulation (320-322). Further, targeted disruption of mouse ADAMTS1 gene results in abnormal adrenal medulla architecture without capillary formation, suggesting a role in angiogenesis (323). ADAMTS1 can cleave the proteoglycans aggrecan and

versican (324,325), which may regulate ovulation (326). Inactivation of the gene by homologous recombination in mice resulted in decreased growth, and renal defects (327). ADAMTS1 has been demonstrated to promote collagen degradation (251), fitting with our data that decreasing ADAMTS1 expression levels resulted in decreased invasion density and distances. ADAMTS1 was first cloned from a colon adenocarcinoma (328) and has been reported to be silenced by hypermethylation in colorectal tumors (329). Of note, the addition of recombinant ADAMTS1 to corneal pocket and chick chorioallantoic angiogenic assays (330) resulted in inhibition of angiogenic responses. ADAMTS1 can diminish VEGFR2 phosphorylation by binding and sequestering VEGF. This binding occurs via the C-terminal thrombospondin motifs (331). Interestingly, ADAMTS1 is processed in this region to release the last two TSP domains. The cleavage event requires metalloproteinases, which may include MMP-2; MMP-8; and MMP-15 (332). It has been proposed that ADAMTS1 could function as an anti-angiogenic molecule through the release of TSP repeats (132) as do other proteins that harbor anti-angiogenic potential such as collagen XVIII and plasminogen (333-335). Thus, presentation of ADAMTS1 on the cell surface or in soluble form may be critical for determining its mechanism of action. Thus far, we have been unable to detect cleavage or liberation of ADAMTS1 from the endothelial surface, consistent with its role as a collagenase during EC invasion.

Notch signaling has long been implicated in angiogenic events. It is well documented that the activation of Notch signaling plays an important role in vascular development (336-339). Leong and colleagues have described that expression of constitutively active Notch4 in human dermal microvascular endothelial cells inhibits endothelial sprouting and migration through collagen but not fibrinogen (340). In addition, Sainson and colleagues have studied Notch inhibition in ECs resuspended in fibrin matrices. By using γ-secretase inhibitors, a dominant-negative form of Notch1 or antisense for Notch1 and Notch4, Notch inhibition resulted in

enlarged sprout diameter but not length through enhanced cell proliferation and extensive sprout branching (341). In contrast, another study indicated that constitutive activation of Notch1 enhanced network and cord formation of human iliac artery ECs (342). Here, we showed that several members of Notch signaling pathway, in particular target genes of Notch were downregulated with time in our 3D invasion system (Table 2, Fig. 5), suggesting that Notch signaling is inhibited during EC invasion. Inhibition of Notch signaling using γ-secretase inhibitor promoted increased numbers of invading ECs, and overexpression of a Notch ligand, Dll4 and two activated Notch receptors, N1ICD and N4ICD, all inhibited the EC invasion responses (Fig. 6). Even though all the above Notch data emerged from *in vitro* 3D models, minor variations in the experimental conditions, such as matrices used, stimuli added, cell types involved and the way Notch signal is manipulated are present among those studies. Paradoxically, the activation of Notch signaling can exhibit both inhibitory effects on angiogenesis (343-346), as well as stimulatory (347-350). There are several variables that may account for these contradictory results. First, signals emitted from *cis* interaction (cell-autonomous activation) could be different from those from *trans* interaction (non-cell-autonomous manner) (351,352). Next, different Notch intracellular domains may contribute to different transcriptional functions and target different genes for expression (353,354). This, therefore, could be the reason that we observed differential effects between activated Notch1 and Notch4 on EC invasion. Another possible explanation is that a conventional two-dimensional cell culture environment fails to accurately reflect how Notch signaling is regulated during EC morphogenesis since sprouting angiogenesis is a 3-D multi-step process. All of above may account for the similarities or discrepancies between our results and other's (340-342).

In this study, we demonstrate that annexin 2 regulates endothelial morphogenesis through an adherens junctions-mediated pathway upstream of Akt. Annexin 2 is a multi-functional

protein and has been reported to be required for cell spreading and adhesion (355,356). In addition, annexin 2 has also been implicated in developmental mechanisms, such as the establishment of epithelial polarity and the formation of new blood vessels (193,194). Since annexin 2 is proposed as a membrane scaffold protein, its functions are commonly ascribed to an ability to associate with plasma membrane, which can be regulated by various factors in different conditions. Intracellular Ca^{2+} mobilization is the primary signal to induce recruitment of annexin 2 to membranes (197). Here, for the first time, we have shown that membrane translocation of annexin 2 is triggered by S1P, which increases intracellular Ca^{2+} levels (33,34). In addition to elevating intracellular Ca^{2+} concentration, other mechanisms have been reported to target annexin 2 to membranes, including tyrosine phosphorylation and cleavage of annexin 2 (356-358). Also, cell density may mediate the membrane translocation of annexin 2 in particular circumstances (199). Considerable evidence has revealed that the specificity for membrane targeting of annexin 2 lies in cholesterol-enriched membrane rafts (357,359). Recently, study of S1P-induced barrier stabilization indicated that annexin 2 was present in membrane rafts as human pulmonary artery endothelial cells were challenged with S1P (360). Our data, together with findings from others suggest that annexin 2 acts downstream of S1P signaling to regulate various cellular responses, including endothelial morphogenesis. Determining the mechanism by which S1P induces recruitment of annexin 2 to membranes will require further investigation.

ECs are interconnected via three distinct junctional structures, including adherens junctions, tight junctions and gap junctions. Adherens junctions and tight junctions are mainly responsible for intercellular adhesion via the formation of actin filament-associated protein complexes along transmembrane adhesion sites (167) and both require the presence of annexin 2 (199,202). Since we showed that a cohort of cell adhesion molecule genes involved in assembly and regulation of adherens junction were upregulated, whereas a set of genes associated with

tight junctions were downregulated, during EC invasion (Fig. 2A), we speculated that the action of annexin 2 on EC sprouting behaviors was associated with adherens junctions. In the present study, we demonstrated a functional role for annexin 2 in adherens junction-related cellular responses, including endothelial permeability on Transwell membranes, along with tyrosine phosphorylation of VE-cadherin and Akt activation during EC invasion. In addition, it is intriguing that annexin 2 depletion augmented tyrosine phosphorylation of VE-cadherin during EC invasion, although the mechanism of VE-cadherin phosphorylation has not yet been fully clarified. This finding may be due to either a decrease in AJ-associated phosphatases recruited by annexin 2 or enhanced kinase activity in the absence of annexin 2. Moreover, we observed increased association of annexin 2 with VE-cadherin following S1P stimulation, suggesting that annexin 2 may play a role in S1P-enhanced endothelial barrier function. S1P signaling not only regulates the localization of VE-cadherin and other catenins at endothelial junctions, but also the expression of these junctional molecules (32,361,362). In this study, similar effects of S1P were observed for expression and localization of annexin 2. Hence, with S1P stimulation, the functional connection of annexin 2 with the integrity of endothelial adherens junctions plays a key role in modulating EC invasion.

We have previously shown that inhibition of Akt activation diminished the EC invasion responses induced by the combined application of fluid shear stress and S1P (363). Akt is critical in the regulation of various EC functions and maintenance of vascular integrity. Here, we demonstrate that knockdown of annexin 2 attenuated Akt activation and invasion responses during EC invasion, as did disrupting the homotypic binding of VE-cadherin. Expression of a constitutively active version of Akt partially, but significantly restored the impairment of invasion responses as well as endothelial barrier functions caused by depletion of annexin 2 or VE-cadherin. This finding is consistent with the proposed role of Akt in vascular maturation and

angiogenesis during wound healing (364), which is mimicked in our 3-D *in vitro* model (365). In addition, Akt was shown to be crucial for the restoration of reactive oxygen species-stressed barrier integrity in human cardiac microvascular ECs (277), which is consistent with our findings that annexin 2 knockdown decreased Akt activation and increased endothelial permeability. However, introducing constitutively active Akt did not fully rescue EC invasion responses blocked by annexin 2 knockdown, while constitutively active Akt almost completely rescued EC invasion responses in VE-cadherin-depleted cells. One explanation for this outcome is that annexin 2 is recognized as a multi-functional protein and implicated in various molecular mechanisms, including (but not limited to) the regulation of actin dynamics as well as endocytic and secretory pathways (198,200,201). The reimbursement of Akt activity is insufficient to compensate completely for the depletion of annexin 2 during EC invasion. This suggests other ancillary roles for annexin 2 in endothelial morphogenesis. Furthermore, Akt modulates various signaling pathways, many of which have been shown to be important for angiogenesis in different scenarios (79). The signaling pathways downstream of annexin 2-mediated Akt activation that control EC morphogenesis remain to be elucidated.

5. CONCLUSIONS

In conclusion, following stimulation by S1P and angiogenic growth factors, bFGF, and VEGF, gene expression profiles during EC invasion in 3D collagen matrices were analyzed. Consistencies exist between our data and findings reported from other *in vitro* models and *in vivo* studies. The validation of gene expression in this study is focused on cell adhesion molecules, ECM, proteases/anti-proteases, Wnt and Notch signaling pathways, and potential regulators of angiogenesis at mRNA or protein levels. In addition, ADAMTS1 shows a functional requirement for ECs to invade, and Notch signal serves as an inhibitory role in EC invasion. This knowledge will serve as a valuable resource for exploring molecular mechanisms underlying sprouting angiogenesis. Moreover, we have demonstrated that annexin 2 modulates endothelial morphogenesis via an adherens junction-mediated pathway upstream of Akt, revealing for the first time that annexin 2 regulates Akt activation during sprouting angiogenesis driven by S1P and angiogenic growth factors. We show that S1P triggered annexin 2 translocation from cytosol to plasma membrane and coupling with VE-cadherin. Knockdown of annexin 2 attenuated Akt activation during EC invasion, as did disrupting homophilic interactions of VE-cadherin. Furthermore, silencing of annexin 2 increased tyrosine phosphorylation of VE-cadherin during EC invasion and decreased junctional integrity, indicated by increased FITC-dextran flux across EC monolayers and decreased TEER. Expression of a constitutively active form of Akt restored the decreased EC invasion and endothelial permeability caused by annexin 2 depletion. Our results provide novel insight into annexin 2 function, which is not only activated downstream of S1P signaling but also linked to the stabilization of adherens junctions.

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APPENDIX A

Primer sequence (5'-3')

HEY1, forward: CTGTCGGCCTTCCCCTTCTC, reverse: CTGACGACTTTAAGGTGATGTTGG

SPP1, forward: GGGACCAGACTCGTCTCAGG, reverse: CTGGGTATTTGTTGTAAAGCTGCT

GADPH, forward: GAACATCATCCCTGCCTCTACTG, reverse: TAGCCCAGGATGCCCTTGAG

DAAM1, forward: AGTTAAGCAGTGGAAAGAACAAGC, reverse: CATGGAGCTGTGCTGTGAGG

DLL4, forward: TGCCTGAACCGAGGTCCAAG, reverse: GATGGATGTCCGCACCTCAC

NID2, forward: AATGATGGCAGAGCCTACACG, reverse: CAGCAGTTTGAGTGATACGAACC

PCDH17, forward: GCTGGGTACAGTGGTCATCG, reverse: AGCATCCCGTTTTCCTCATAGTC

PODXL, forward: GCTGCTGCTACTGTTGTCAAC, reverse: TTCGTTGGCCTTGGAAGTGG

NFKBIa, forward: TGGGAGTCCTGACTCAGTCC, reverse, CAGGATTTTGCAGGTCCACTG

HHIP, forward: ATGCAAGAAAAGATGGTGGGTTG, reverse: CCACTATGCAGGGCACCAAC

CLDN11, forward: CCGGTGTGGCTAAGTACAGG, reverse: CGCAGCAGAGGATGACACAG

ADAMTS1, forward: CCACCAACATCGAAGTGAAACAG, reverse: AGAGGGCTAAAGCTGCGAATTC

ADAMTS5, forward: GTAAACTCTGTCACTAGTCATGGC, reverse: GCCTTTGGGAGAGAGGACATC

AXUD1, forward: TGCTTCCTGGAGTCCCTCATG, reverse: GGAGCCCCATAATTACAAGAAAGC

DKK2, forward: CGATCATCAGACTGCATTGAAGG, reverse: TAGGTGGCATCTTTCCATACTTTG

FZD4, forward: GTGCTCAAGTGTGGCTATGATG, reverse: TCAGCCTGACAATATAAGCAATGC

LXN, forward: CCTGAAGTCACTGCTCATCCG, reverse: AAGGCGATACTTATGTCCTCTTCC

SELE, forward: AAATCCCAGTTTGTGAAGCTTTCC, reverse: GCTTCTCGTTGTCCCACTCC

ITGA2, forward: TCGGAGCAATTCAATATGCAAG, reverse: GGGCGTTTCTGTTTAAGTACC

ITGAV, forward: CCCCGAGGGAAGTTACTTCG, reverse: ATGGATCATCCTTGGCATAATCTC

ANTXR2, forward: GATCTACTGAGGAAGGTGCAAGG, reverse: TGAGGTCGCATCAAAGAAACCC

PCDH10, forward: AGCTCAACATCTATACTTGTCTGG, reverse: GACTCCTCTATCGGCACCTG

NOX4, forward: CCCACCCTCCCGGCTGCATC, reverse: GAAATCCAAAGCCAAGTCTGTG

APPENDIX B

shRNA sequences :

For gene silencing, HUVECs were infected with pLKO.1-puro lentiviral vectors encoding shRNAs against human annexin A2, β2-microglobulin, GFP, VE-cadherin, and PECAM1 (Sigma). For annexin 2 or VE-cadherin knockdown, findings were reproduced by two distinct shRNAs.

Annexin A2 :

CCGGGCAGGAAATTAACAGAGTCTACTCGAGTAGACTCTGTTAATTTCCTGCTTTTTG

CCGGCGGGATGCTTTGAACATTGAACTCGAGTTCAATGTTCAAAGCATCCCGTTTTTG

β2-microglobulin :

CCGGCCGTGTGAACCATGTGACTTTCTCGAGAAAGTCACATGGTTCACACGGTTTTTG

CCGGCCCAAGATAGTTAAGTGGGATCTCGAGATCCCACTTAACTATCTTGGGTTTTTG

GFP :

CCGGTACAACAGCCACAACGTCTATCTCGAGATAGACGTTGTGGCTGTTGTATTTTTG

VE-cadherin :

CCGGCGCCTCTGTCATGTACCAAATCTCGAGATTTGGTACATGACAGAGGCGTTTTTG

CCGGCGTGGATTACGACTTCCTTAACTCGAGTTAAGGAAGTCGTAATCCACGTTTTTG

PECAM1 :

CCGGCGGAGTGATCATTGCTCTCTTCTCGAGAAGAGAGCAATGATCACTCCGTTTTTG

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